CA 04 / 1470

Office de la propriété intellectuelle du Canada
Un organisme d'Industrie Canada

Canadian Intellectual Property Office
An Agency of Industry Canada

REC'D 03 NOV 2004
WIPO PCT

CA04/01470

Bureau canadien des brevets
Certification

La présente atteste que les documents ci-joints, dont la liste figure ci-dessous, sont des copies authentiques des documents déposés au Bureau des brevets.

Canadian Patent Office
Certification

This is to certify that the documents attached hereto and identified below are true copies of the documents on file in the Patent Office.

Specification and Drawings, as originally filed, with Application for Patent Serial No: **2,436,650**, on August 6, 2003, by **NATURIA INC.**, assignee of Jean-Michel Garro and Paul Angers, for "Conjugated Linolenic Acid (CLnA™) Compositions: Synthesis, Purification and Uses".

PRIORITY DOCUMENT
SUBMITTED OR TRANSMITTED IN COMPLIANCE WITH RULE 17.1(a) OR (b)

Agent certificateur/Certifying Officer

September 21, 2004
Date

Canada

OPIC CIPO

Synthesis and anti-tumor activity of CLnA™

Abstract

The invention relates to the discovery that CLnA™ compounds induce apoptosis of cancer cells. The activity of CLnA™ was demonstrated in two human breast cancer lines (breast cancer cells MCF-7 and MDA-MB-231), using MTT assay and fluorescence-based assay. Our results suggest that CLnA™ has a cytotoxic activity and induce apoptosis in human solid tumors cells lines. Therefore may be used for the treatment of cancer, including advanced cancer.

References cited (Patent documents)

JP2000336029	December 2000	Tomoyuki *et al.*
6,245,811	June 2001	Horrobin *et al.*
6,319,950	November 2001	Seidel *et al.*
US2002077317	June 2002	Das *et al.*
6,426,367	July 2002	Das *et al.*
CN1371985	July 2002	Bin *et al.*
CN1356386	July 2002	Bin *et al.*
WO02051255	July 2002	Focant *et al.*

Other References

Delany JP, West DB (2000) Changes in body composition with conjugated linoleic acid. *J Am Coll Nutr* 19(4):487S-493S.

Destaillats *et al.* (2003) Evidence for a conjugated linolenic acid (CLnA™) in milk fat: *cis*-9, *trans*-11, *cis*-15. *Lipids* In press.

Durgam, VR, Fernandes G (1997) The growth inhibitory effect of conjugated linoleic acid on MCF-7 cells is related to estrogen response system. *Cancer Lett* 116:121-130.

Futakuchi m, Cheng JL, Hirose M, Kimoto N, Cho YM, Iwata T, Kasai M, Tokudome S, Shirai R (2002) Inhibition of conjugated fatty acids derived from safflower or perilla oil of induction and development of mammary tumors in rats induced by 2-amino-1-methyl-6-phenylimidazol[4,5-b]pyridine (PhIP). *Cancer Lett* 178:131-139.

Ha YL, Grimm NK, Pariza MW (1987) Anticarcinogens from fried ground beef: heat-altered derivatives of linoleic acid *Carcinogenesis* 8:1881-1887.

Harris WS, Park Y, Isley Wl (2003) Cardiovascular disease and long-chain omega-3 fatty acids. *Curr Opin Lipidol* 14:9-14.

Conjugated Linolenic Acid (CLnA™) compositions: synthesis, purification and uses

FIELD OF THE INVENTION

The invention relates to the field of human and animal nutrition, and more particular to novel compositions of conjugated linolenic acids compounds (CLnA™: a 1:1 mixture of C18:3 9*cis*,11*trans*,15*cis*-octadecatrienoic acid and C18:3 9*cis*,13*trans*,15*cis*-octadecatrienoic acid) and their derivatives (salts, amides, esters, adducts and complexes). More precisely, the invention relates to the process for their production and its use for prevent and/or treat different deceases like cancer, obesity, diabetes, atherosclerosis, cerebral vascular disease (CVD) and all related coronary artery deceases. These compositions are prepared from materials rich in alpha or gamma linolenic acids like linseed oil or evening primrose oil and more particularly from a new natural source *Plukenetia volubilis* (Sacha Inchi or Inca Peanut), a native plant of the high altitude rain forests of the Andean region of South America. According to this process, only water is used as a solvent for isomerisation with a metal alkali (i.e NaOH, KOH, $Ca(OH)_2$) as catalyst.

When linseed oil is used as starting material for execution of the present invention, the reaction produces approximately 30% of a 1:1 mixture of C18:3 isomers: 9*cis*,11*trans*,15*cis*-octadecatrienoic acid and 9*cis*,13*trans*,15*cis*-octadecadienoic; 9.5% of saturated fatty acids (5.4% palmitic and 4.3% stearic). The isomerised oil also contains 20% of unreacted oleic acid, 13% of unreacted linoleic acid (C18:2 9*cis*, 12*cis*); 4 % of CLA wherein 1.6% accounts for C18:2 9*cis*, 11*trans* and 2.3 % for C18:2 10*trans*, 12*cis*. The isomerised oil also contains 9% of unreacted linolenic acid (C18:3 9*cis*, 12*cis*, 15*cis*). All other full conjugated C18:3 compounds accounts for 9% and the cyclic compound C18:3 11,13 ciclohexadiene accounts for 6.7 %.

When *Plukenetia volubilis* (Sacha inchi) oil is used as starting material for execution of the present invention, the reaction also produces approximately 30% of a 1:1 mixture of C18:3 isomers: 9*cis*,11*trans*,15*cis*-octadecatrienoic acid and 9*cis*,13*trans*,15*cis*-octadecadienoic. Oleic

acid content (9.75%) is comparable to that obtained with linseed oil but it has less saturated fatty acids (4.16% palmitic and 3% stearic). The main difference concerns the CLA content (24%) where 11.6% accounts for C18:2 9*cis*, 11*trans* and 12.4% for C18:2 10*trans*, 12*cis*. The isomerised oil also contains 6.8% of unreacted linoleic acid (C18:2 9*cis*, 12*cis*); and only 0.38% of unreacted linolenic acid (C18:3 9*cis*, 12*cis*, 15*cis*). All other full conjugated C18:3 compounds accounts for 12.4% and the cyclic compound C18:3 11,13 ciclohexadiene accounts for 7.5 %.

Purification of the isomerised oil by urea crystallization enables to separate many different fractions one of them rich in cyclic compounds (68%) and other with the desired 1:1 mixture of C18:3 isomers (75%). Preparative chromatography was used to purify this mixture until 90%.

BACKGROUND OF THE INVENTION

Processes for the conjugation of double bonds on polyunsaturated fatty acids are well known in the previous art. They were used in the 40's to improve the polymerization properties of the synthetic polyhydric alcohol esters obtained by esterification of polyhydric alcohols with fatty acid mixtures of vegetable oils.

US patent 2,350,583 and British patent 558,881 claim a method to produce fatty acids capable of forming polyhydric alcohol esters by heating a solution of linseed fatty acid soaps with an excess of alkali in water in the absence of an added alcohol for several hours at temperature ranges of 200°C – 250°C. The product was found to contain 34.6% of 9*cis*, 11*trans*-octadecadienoic acid and 7.66% of 10,12,14 octadecatrienoic acid. At these high temperatures the sigmatropic rearrangement conducing to cyclic compounds occurs very fast and most of the linolenic acid present in linseed oil is consumed in the formation of these products. Probably the unreasonable high concentration described for C18:2 9*cis*,11*trans* (34.6%) could be better understand in terms of this kind of cyclic compounds formation and the unavailable pertinent analytical methods at that time. There is only 16% of linoleic acid (C18:2 9*cis*, 12cis) in linseed oil available for conversion to C18:2 9*cis*, 11*trans* and/or 10*trans*, 12*cis* as a matter of fact, it's impossible to obtain 34.6% of only one isomer C18:2 9*cis*, 11*trans*.

The conjugation of double bonds on polyunsaturated fatty acids was principally applied to the preparation of conjugated linoleic acid (CLA), a 1:1 mixture of C18:2 *9cis*, 11*trans* and C18:2 10*trans*, 12*cis*. US patents 2,389,260; 2,242,230; 2.343,644 describe the formation of fatty acids soaps and their isomerisation with strong alkali bases in molten soaps, alcohol and ethylene glycol, followed by acidification with strong acid and repeatedly washings with brine or $CaCl_2$. More recently US patent 6,420,577 describes a process for producing a CLA rich fatty acid mixture by reacting a linoleic acid-rich oil with a catalytic amount of base in an aqueous medium containing up to 100 moles of water per mole of hydrolysable acyl group at temperatures above 170°C. This reaction mixture is cooled to 90°C - 100°C and acidified to convert the soaps into the desired acids. A demulsifier is used to facilitate the separation. The linoleic acid-rich oils claimed for the process are cottonseed, cucumber, grapeseed, corn, safflower, soybean, sunflower, high linoleic acid flaxseed oil and walnut oil. The same patent describes a test tube experience for the conjugation of pure linolenic acid (via its methyl ester) with $KOCH_3$ in methanol at 120°C for 3 hours. Analytical results shown seven unknown isomers.

Main difference between all these procedures and the present invention is the fact that linolenic acid has three double bounds (*9cis*, 12*cis*, 15*cis*) that are much more reactive than the two double bonds of linoleic acid (*9cis*, 12*cis*). More precisely, the octatrienoic system (C18:3) is responsible for a sigmatropic rearrangement (see Fig. 1) that conduces to the formation of cyclic compounds (C18:3 11,13 cyclohexadiene) that are not possible to be formed during the isomerisation of the octadienoic system (C18:2). A rigorous control of the reaction kinetic's was necessary to maximize the yield of the desire mixture of isomers and minimize the amount of cyclic compounds. In fact, purification steps used in this invention are set in order to separate these cyclic compounds. Another issue of the purification of the isomerised oil obtained by this process is the elimination of saturated fatty acids. Still another issue of the purification is the isolation of an inexpensive rich fraction of cyclic compounds (C18:3 11,13 cyclohexadiene) that could be use as a synthon in chemical reactions.

In the development of commercial compounds of CLnA™ it is important to have an inexpensive process to produce specific compositions that could be used in different formulations like nutritional bars and beverages, yoghurts, ice creams, cheese, butter, etc.

SUMMARY OF THE INVENTION

The invention relates to the field of human and animal nutrition, and more particular to novel compositions of conjugated linolenic acids compounds (CLnA™: a 1:1 mixture of C18:3 9*cis*,11*trans*,15*cis*-octadecatrienoic acid and C18:3 9*cis*,13*trans*,15*cis*-octadecatrienoic acid) and their derivatives like triglycerides, diglycerides, monoglycerides, salts, amides, esters, adducts and complexes. More precisely, the invention relates to the process for its production and its use for prevent and/or treat different deceases like cancer, obesity, diabetes, atherosclerosis, cerebral vascular disease (CVD) and all related coronary artery deceases.

Definitions

Concerning CLA:

- General term used to describe octadienoic acid systems C18:2 (18 carbons, 2 insaturations).
- Commercial term used to described a 1:1 mixture of C18:2 9*cis*, 11*trans* and C18:2 10*trans*, 12*cis*. Concentrations for the mixture may vary between 30% and 90%.
- Linoleic acid (C18:2 9*cis*, 12*cis)*, the major fatty acid present in different vegetal oils (sunflower, safflower, soya, corn, etc) used as starting material for CLA production. Regarding its chemical structure, it could be also considered as a CLA.

Concerning CLNA:

- General term used to describe octatrienoic acid systems C18:3 (18 carbons, 3 insaturations).
- Linolenic acid (C18:3 9*cis*, 12*cis*, 15*cis)*, the major fatty acid present in different vegetal oils (linseed, basil, *Plukenetia volubilis*, etc) used as starting material for CLnA™ production. Regarding its chemical structure, it could be also considered as a CLNA.

Concerning CLnA™

- Commercial term used by Naturia Inc. to described a 1:1 mixture of C18:3 isomers: 9*cis*,11*trans*,15*cis*-octadecatrienoic acid and 9*cis*,13*trans*,15*cis*-octadecadienoic. Concentrations for the mixture may vary between 30% and 90%. Thus, the nomenclature for these products will be represented by CLnA™ – 30 and CLnA™ – 90 respectively.

The present invention describes a process for the preparation of CLnA™ compositions (Fig. 2). These compositions are characterized by the fact that, even though the mixture can be obtained at different concentrations (i.e. 30%, 40%, 45%, 75% and 90%) among the purification process, the ratio between both isomers remains constant at 1:1.
According to the present invention, only water is used as a solvent for isomerisation with a metal alkali (i.e NaOH, KOH, $Ca(OH)_2$) as catalyst. Preferred range for each reagent is as follows:

8 – 25%	Linseed or *Plukenetia volubilis* oils
70 – 90%	Propylene Glycol or water
3 – 7 %	NaOH or KOH

When linseed oil is used as starting material for execution of the present invention (Table N 10: essays # 0 to 8 for reaction parameters and Tables 1 to 8 for analytical results), the reaction produces approximately 30% of a 1:1 mixture of C18:3 isomers: 9*cis*,11*trans*,15*cis*-octadecatrienoic acid and 9*cis*,13*trans*,15*cis*-octadecadienoic; 9.5% of saturated fatty acids (5.4% palmitic and 4.3% stearic). The isomerised oil also contains 20% of unreacted oleic acid, 13% of unreacted linoleic acid (C18:2 9*cis*, 12*cis*); 4 % of CLA where 1.6% accounts for C18:2 9*cis*, 11*trans* and 2.3 % for C18:2 10*trans*, 12*cis*. The isomerised oil also contains 9% of unreated linolenic acid (C18:3 9*cis*, 12*cis*, 15*cis*). All other full conjugated C18:3 compounds accounts for 9% and the cyclic compound C18:3 11,13 ciclohexadiene accounts for 6.7 %.

When *Plukenetia volubilis* (Sacha inchi) oil is used as starting material for execution of the present invention material (Table N 10: essays 9 for reaction parameters and Tables 9 for analytical results), the reaction also produces approximately 30% of a 1:1 mixture of C18:3 isomers: 9*cis*,11*trans*,15*cis*-octadecatrienoic acid and 9*cis*,13*trans*,15*cis*-octadecadienoic. Oleic

acid content (9.75%) is comparable to that obtained with linseed oil but it has less saturated fatty acids (4.16% palmitic and 3% stearic). The main difference concerns the CLA content (24%) where 11.6% accounts for C18:2 9*cis*, 11*trans* and 12.4% for C18:2 10*trans*, 12*cis*. The isomerised oil also contains 6.8% of unreacted linoleic acid (C18:2 9*cis*, 12*cis*); and only 0.38% of unreacted linolenic acid (C18:3 9*cis*, 12*cis*, 15*cis*). All other full conjugated C18:3 compounds accounts for 12.4% and the cyclic compound C18:3 11,13 ciclohexadiene accounts for 7.5 %.

In both cases, purification was performed under a rigorous control of temperature, time and the ratio between the oil, the urea and methanol. Repeatedly purification by urea crystallization enables to separate a rich fraction of cyclic compounds (67.75 % in Table N 11: Urea 3 Liquid fraction) and raise the concentration of the desired 1:1 mixture of C18:3 isomers to more than 75% (Table N 11: Urea 4 Solid fraction). Preparative chromatography was used to purify this mixture until 90%. Gas chromatography analysis has shown the presence of both isomers (Fig. 3).

This process uses materials rich in alpha linolenic acid (i.e. linseed oil) or gamma linolenic acids (i.e. evening primrose oil) and more particularly a new natural source rich in alpha linolenic acid *Plukenetia volubilis* (Sacha Inchi or Inca Peanut), a native plant of the high altitude rain forests of the Andean region of South America.

BRIEF DESCRIPTION OF FIGURES

Fig. 1: Sigmatropic rearrangement leading to C18:2 11,13 ciclohexadiene.

Fig. 2: Diagram flow of the invention.

Fig. 3: Gas chromatography of CLnA™ at 30% purity for the 1:1 mixture of C18:3 isomers 9*cis*,11*trans*,15*cis*-octadecatrienoic acid and 9*cis*,13*trans*,15*cis*-octadecadienoic acids.

DETAILED DESCRIPTION OF THE INVENTION

The present invention describes a process to manufacture CLnA™ compositions at different concentrations (Fig 2). Table N 10 summarizes different conditions used to perform the saponification and isomerisation over triglycerides present in linseed oil (essays # 1,2,3,4,5,7,8) and *Plukenetia volubilis* (essay #9) using water as a solvent and NaOH as a base catalyst. Essay # 0, in Table N 10, presents the conditions used for isomerisation with propylene glycol as solvent and NaOH as catalyst.

According to the process described in the present invention, preferred range for each reagent is as follows:

8 – 25%: Linseed or *Plukenetia volubilis* oils

70 – 90%: Propylene Glycol or water

3 – 7 %: NaOH or KOH

EXPERIMENTAL

The following examples are provided in order to demonstrate and further illustrate certain preferred embodiments and aspects of the present invention. It should be understood that the invention as claimed should not be limited to such specific embodiments. Modifications of the described process for those skilled in the art are intended to be within the scope of the present invention.

In the experimental disclosure which follows, the following abbreviations apply: Kg (kilograms); g (grams); mg (milligrams); °C (degrees centigrade); L (liters); mL (milliliters); µL (microliters); m (meters); cm (centimeters); mm (millimeters), µm (micrometers); NaOH (sodium hydroxide), H_2SO_4 (sulfuric acid), NaCl (sodium chloride); C18:2 11,13 ciclohexadiene (cyclic compounds).

EXAMPLE 1: Linseed oil isomerisation in propylene glycol.

In a preferred embodiment 378 gr of NaOH were dissolved in 7778 kg of propylene glycol at 160C in a 25 L stainless steel reactor with a condenser. When dissolution was completed (30

min) 712 g of linseed oil were loaded under vacuum and nitrogen was use to reestablish the atmospheric pressure. The reaction was performed under nitrogen atmosphere at 160°C during 2 hours. (Table N10: Assay #0). After what, the mixture was cooled to 25°C and pH was adjusted to 3 with 460 g of concentrated H_2SO_4 dissolved in 7.61 Kg of water. After 15 minutes decantation the aqueous phase was removed and 45 Kg of water were added to the reactor to wash the isomerised fatty acid oily phase. After another 15 min decantation the washing water was removed to obtain 655 g of the isomerised linseed oil that was analyzed by gas chromatography by the method previously described. The fatty acids profile for the isomerised product is described in Table 1 at the column "Propylene glycol". It has 30.94% of a 1:1 mixture of C18:3 isomers: 9*cis*,11*trans*,15*cis*-octadecatrienoic acid and 9*cis*,13*trans*,15*cis*-octadecadienoic. Under our nomenclature it is named CLnA™ – 30. As a reference, the column "Linseed oil" in Table N1 presents the fatty acids profile of this particular starting material. It is clear that almost all the 53.53% of the linolenic acid (C18:2 9*cis*, 11*cis*, 15*cis*) present in linseed oil was reacted (only 0.22% was not reacted) to produce 30.94% of the desired mixture, 8.32% of the cyclic compounds, and 11.57% of full conjugated C18:3 isomers. Regarding the distribution of C18:3 isomers the corresponding yields of conversion were: 60.87%, 13.67% and 22.76% respectively.

First urea crystallization was performed over the 655 g of CLnA™ – 30 obtained in the saponification/isomerisation step. A methanolic-urea solution was prepared by dissolving 1.3 Kg of urea with 4.140 Kg of methanol at reflux temperature in a stainless steel reactor. Once all the urea dissolved, 655 g of CLnA™ – 30 were added to the reactor under agitation. The reaction mixture was cooled until 25°C in 10 minutes and then cooled to 25°C in 10 hours. After what the urea adduct was filtrated over a previously cooled centrifuged to separate a solid and a liquid fractions. The liquid phase was decomplexed by addition of 98 g of concentrated H_2SO_4 dissolved in 10.6 Kg of water (approximately a 1% w/w H_2SO_4 solution). After decantation, the aqueous phase was removed and the oily phase washed with a 5% w/w NaCl aqueous solution (270 gr of NaCl in 5.12 Kg of water) to obtain 393 g of 1st purified isomerised linseed oil. The product was analyzed by gas chromatography by the method previously described.
The composition of the Urea 1 Liquid (U1L) product was shown in Table 11 at the column U1L (1st column shadowed). The concentration of the desired 1:1 mixture of C18:3 isomers:

9*cis*,11*trans*,15*cis*-octadecatrienoic acid and 9*cis*,13*trans*,15*cis*-octadecadienoic was 39.96%. Under our nomenclature it is named CLnA™ – 40.

Second urea crystallization was performed over the 393 g of CLnA™ – 40 obtained in the 1st urea crystallization step (U1L). A methanolic-urea solution was prepared by dissolving 1.572 Kg of urea with 4.97 Kg of methanol at reflux temperature in a stainless steel reactor. Once all the urea dissolved, 393 g of CLnA™ – 40 were added to the reactor under agitation. The reaction mixture was cooled until 25°C in 10 minutes and then cooled to 25°C in 8 hours. After what the urea adduct was filtrated over a previously cooled centrifuged to separate a solid and a liquid fractions. The liquid phase was decomplexed by addition of 29.4 g of concentrated H_2SO_4 dissolved in 3.166 Kg of water (approximately a 1% w/w H_2SO_4 solution). After decantation, the aqueous phase was removed and the oily phase washed with a 5% w/w NaCl aqueous solution (162 gr of NaCl in 2.76 Kg of water) to obtain 236.4 gr of 2nd purified isomerised linseed oil. The product was analyzed by gas chromatography by the method previously described.
The composition of the Urea 2 Liquid (U2L) product was shown in Table 11 at the column U2L (2nd column shadowed). The concentration of the desired 1:1 mixture of C18:3 isomers: 9*cis*,11*trans*,15*cis*-octadecatrienoic acid and 9*cis*,13*trans*,15*cis*-octadecadienoic was 45.4%. Under our nomenclature it is named CLnA™ – 45.

Third urea crystallization was performed over the 236.4 g of CLnA™ – 45 obtained in the 2nd urea crystallization step (U2L). A methanolic-urea solution was prepared by dissolving 946 g of urea with 2.9 Kg of methanol at reflux temperature in a 5 L three necked-flask. Once all the urea dissolved, 236.4 g of CLnA™ – 45 were added to the flask under agitation. The reaction mixture was cooled until 25°C in 10 minutes and then cooled to 25°C in 6 hours. After what the urea adduct was filtrated over a previously cooled büchner to separate a solid and a liquid fractions. The solid phase was decomplexed by addition of 17.71 g of concentrated H_2SO_4 dissolved in 19 Kg of water (approximately a 1% w/w H_2SO_4 solution). After decantation, the aqueous phase was removed and the oily phase washed with a 5% w/w NaCl aqueous solution (97.3 gr of NaCl in 1.85 Kg of water) to obtain 28.5 g of 3nd purified isomerised linseed oil. The product was analyzed by gas chromatography by the method previously described.

The composition of the Urea 3 Solid (U3S) product was shown in Table 11 at the column U3S (3rd column shadowed). The concentration of the desired 1:1 mixture of C18:3 isomers: 9*cis*,11*trans*,15*cis*-octadecatrienoic acid and 9*cis*,13*trans*,15*cis*-octadecadienoic was 72.34%. Under our nomenclature it is named CLnA™ – 70.

Fourth urea crystallization was performed over the 28.5 g of CLnA™ – 70 obtained in the 3rd urea crystallization step (U3S). A methanolic-urea solution was prepared by dissolving 57 g of urea with 180 g of methanol at reflux temperature in a 500 mL three necked-flask. Once all the urea dissolved, 28.5 g of CLnA™ – 70 were added to the erlenmeyer under agitation. The reaction mixture was cooled until 25°C in 10 minutes and then cooled to 25°C in 6 hours. After what the urea adduct was filtrated over a previously cooled büchner filter to separate a solid and a liquid fractions. The solid phase was decomplexed by addition of 2.13 g of concentrated H_2SO_4 dissolved in 230 g of water (approximately a 1% w/w H_2SO_4 solution). After decantation, the aqueous phase was removed and the oily phase washed with a 5% w/w NaCl aqueous solution (11.7 g of NaCl in 222.6 g of water) to obtain 21.36 g of 4th purified isomerised linseed oil. The product was analyzed by gas chromatography by the method previously described.
The composition of the Urea 3 Solid (U4S) product was shown in Table 11 at the column U4S (4th column shadowed). The concentration of the desired 1:1 mixture of C18:3 isomers: 9*cis*,11*trans*,15*cis*-octadecatrienoic acid and 9*cis*,13*trans*,15*cis*-octadecadienoic was 75.35%. Under our nomenclature it is named CLnA™ – 75.

EXAMPLE 2: Linseed oil isomerisation in water.

In a preferred embodiment 666g of NaOH were dissolved in 15.794 kg of water at 80C in a 25 L stainless steel reactor with a condenser. When dissolution was completed (30 min) 1.428 Kg of linseed oil were loaded under vacuum and nitrogen was use to reestablish the atmospheric pressure. The reaction was performed under nitrogen atmosphere at 170°C during 3 hours. (Table N10: Assay #2). After what, the mixture was cooled to 60°C and a stoichiometric amount of $CaCl_2$ was added under very low agitation. The sodium soaps were transformed into calcium soaps and they precipitate while the sodium chloride formed is solubilized in the aqueous phase

(Fig 2). Calcium soaps of isomerized linseed oil were separated by filtration over a centrifuge and washed with water. The washed calcium soaps were transferred to another reactor containing a stoichiometric amount of H_2SO_4 in methanol. Acidification until pH 3 produces a white precipitate of $CaSO_4$ that was separated by filtration over a Sparkler filter. The solution contains the free fatty acids of the isomerized linseed oil with the composition described in Table 2 after 3 hours reaction. The isomerized oil contains 29.64% of a 1:1 mixture of C18:3 isomers: 9*cis*,11*trans*,15*cis*-octadecatrienoic acid and 9*cis*,13*trans*,15*cis*-octadecadienoic. Under our nomenclature it is named CLnA™ – 30. As a reference, the column "Linseed oil" in Table N2 also presents the fatty acids profile for this starting material. It can be noted that that 10% of the linolenic acid (C18:2 9*cis*, 11*cis*, 15*cis*) present in linseed oil was not reacted. The other fatty acids contained in the isomerised oil are: 6.47 % of the cyclic compounds, and 6.69 % of full conjugated C18:3 isomers. The content of CLA (3.02%) is distributed by 1.66% of C18:2 9*cis*, 11*trans* and 2.06% of C18:2 10*trans*, 12*cis*. Most of the linoleic acid (C18:2 9*cis*, 12*cis*) remains unreacted (13.12 %). The nomenclature for the isomerised oil correspond to CLnATM – 30 and the purifications steps with this corresponding yields and concentrations (via repetitive urea crystallizations) are similar to those used and obtained in example 1.

EXAMPLE 3: *Plukenetia volubilis* oil isomerisation in water.

In a preferred embodiment 1.22 Kg of NaOH were dissolved in 15.508 Kg of water at 80C in a 25 L stainless steel reactor with a condenser. When dissolution was completed (30 min) 491 g of *Plukenetia volubilis* oil were loaded under vacuum and nitrogen was use to reestablish the atmospheric pressure. The reaction was performed under nitrogen atmosphere at 180°C during 4 hours. (Table N10: Assay #9). After what, the mixture was cooled to 60°C and a stoichiometric amount of $CaCl_2$ was added under very low agitation. The sodium soaps were transformed into calcium soaps and they precipitate while the sodium chloride formed is solubilized in the aqueous phase (Fig 2). Calcium soaps of isomerized *Plukenetia volubilis* oil were separated by filtration over a centrifuge and washed with water. The washed calcium soaps were transferred to another reactor containing a stoichiometric amount of H_2SO_4 in methanol. Acidification until pH 3 produces a white precipitate of $CaSO_4$ that was separated by filtration over a Sparkler filter. The

solution contains the free fatty acids of the isomerized *Plukenetia volubilis* oil with the composition described in Table 9. The isomerized oil contains 30.08 % of a 1:1 mixture of C18:3 isomers: 9*cis*,11*trans*,15*cis*-octadecatrienoic acid and 9*cis*,13*trans*,15*cis*-octadecadienoic. Under our nomenclature it is named CLnA™ – 30. As a reference, the column "Linseed oil" in Table N9 also presents the fatty acids profile for this starting material. . It is clear that almost all the 51.82 % of the linolenic acid (C18:2 9*cis*, 11*cis*, 15*cis*) present in *Plukenetia volubilis* oil was reacted (only 0.38 % was not reacted) to produce 30.08 % of the desired mixture, 7.58 % of the cyclic compounds, and 12.41 % of full conjugated C18:3 isomers. Regarding the distribution of C18:3 isomers the corresponding yields of conversion were: 60.08%, 15.14% and 24.79 % respectively. Almost the same fatty acids profile of the example 1. The main difference concerns the much significative quantity of CLA (24%) where 11.6% accounts for C18:2 9*cis*, 11*trans* and 12.4% for C18:2 10*trans*, 12*cis*. %). The nomenclature for the isomerised oil correspond to CLnATM – 30 and the purifications steps with this corresponding yields and concentrations (via repetitive urea crystallizations) are similar to those used and obtained in example 1.

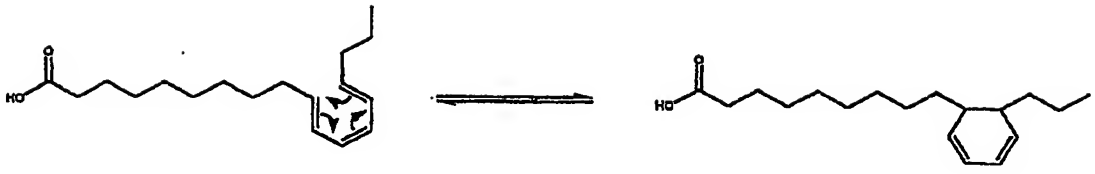
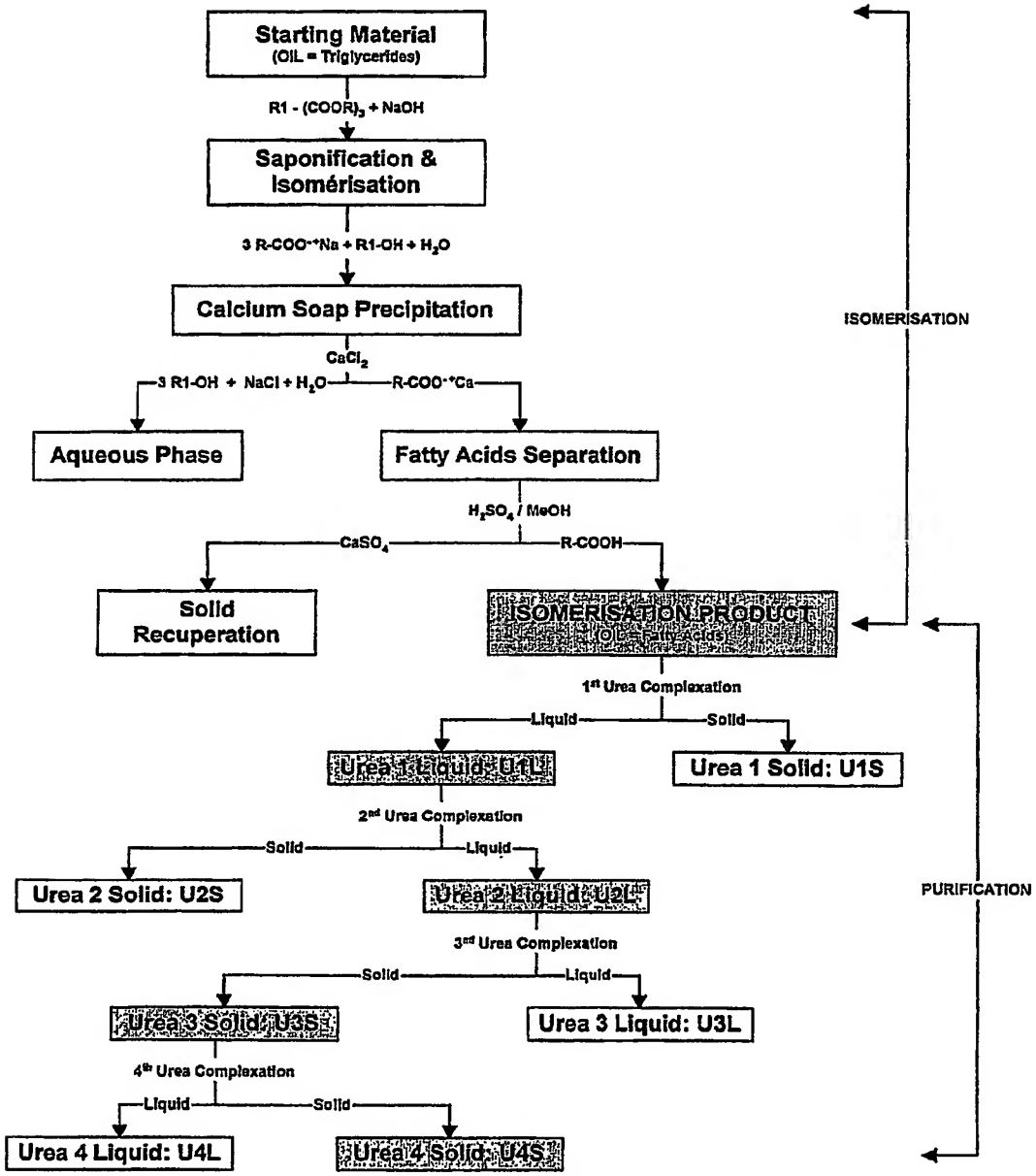


Fig. 1: Sigmatropic rearrangement of octatrienoic system leading to C18:2 11,13 ciclohexadiene.

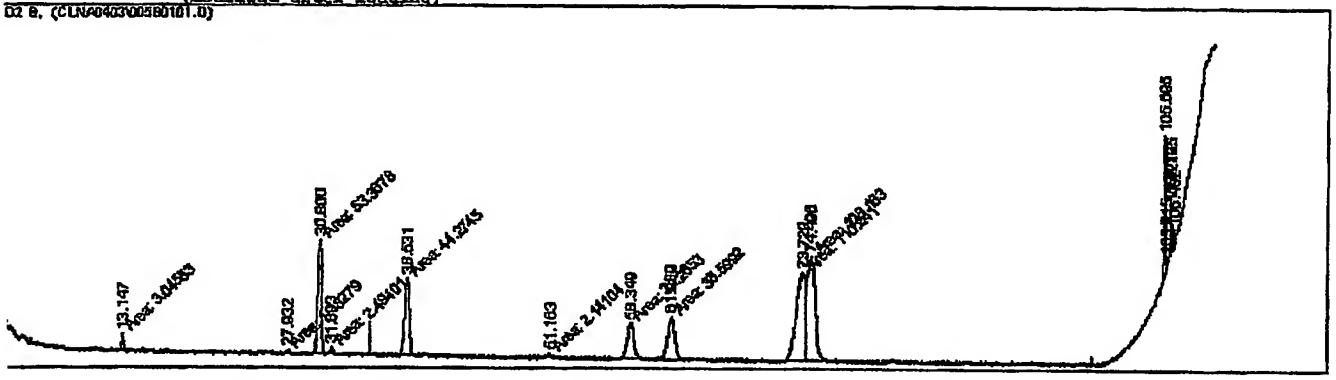
Figure 2: Diagram flow of the invention



File C:\HPCHEM\1\DATA\CLNA0403\005B0101.D Sample Name: U4S

Injection Date	: 03/04/2003 5:04:16 PM	Seq. Line :	1
Sample Name	: U4S	Vial :	5
Seq. Operator	: JP	Inj :	1
		Inj Volume :	1 µl
Seq. Method	: C:\HPCHEM\1\METHODS\IS0120B.M		
Last changed	: 03/04/2003 5:08:05 PM by JP (modified after loading)		
Analysis Method	: C:\HPCHEM\1\METHODS\CL25M180.M		
Last changed	: 07/31/2003 11:01:03 AM by JP (modified after loading)		

FID2 B, (CLNA0403\005B0101.D)



Area Percent Report

Sorted By	:	Signal
Multiplier	:	1.0000
Dilution	:	1.0000

Signal 1: FID2 B,

Peak #	RetTime [min]	Type	Width [min]	Area [pA*s]	Height [pA]	Area %
1	13.147	MM T	0.1396	3.04583	3.63752e-1	0.72288
2	27.932	MM T	0.2727	1.18279	7.22833e-2	0.28071
3	30.800	MM T	0.3590	53.36781	2.47783	12.66594

07/31/2003 11:01:47 AM JP

Table N1: Fatty acids composition for the isomerized oil at different times in assay #1

Fatty Acids	Linseed Oil	ISOMERISATION: Assay #1							
		Propylene glycol		Water					
				T = 160C	t = 1h	T = 180C	t = 1,5h	T = 180C	t = 2h
Saturated									
C16:0	5.40	5.53		5.25		5.54		5.56	
C18:0	4.13	4.26		4.09		3.93		4.28	
Total Saturated		9.79		9.34		9.47		9.84	
Monoenes									
C18:1	19.77	21.19		20.48		20.68		21.38	
Dienes									
C18:2 9c, 11t	0.00	5.59		0.27		1.17		1.66	
C18:2 9c, 12c	16.47	5.36		15.91		14.37		13.12	
C18:2 10t, 12c	0.00	5.60		0.48		1.46		2.06	
Total C18:2	16.47	16.55		16.66		17.00		16.84	
Isomerisation C18:2 (conjugated / total)	0.00	**67.61%**		4.50%		15.47%		**22.09%**	
Trienes									
C18:3 9c, 11t, 15c et C18:3 9c, 13t, 15c	0.00	**30.94**	60.87%	9.88	77.37%	27.65	72.01%	**29.64**	66.89%
C18:3 9c, 12c, 15c	**53.53**	**0.22**		39.94		13.78		**6.86**	
C18:2 11, 13 cyclohexadiene	0.00	8.32	16.37%	0.61	4.78%	5.44	14.17%	7.40	16.70%
C18:3 conjugated	0.00	11.57	22.76%	2.28	17.85%	5.31	13.83%	7.27	16.41%
Total	53.53	51.05		52.71		52.18		51.17	
Isomerisation C18:3 (conjugated/ total)	0.00	**99.57%**		24.23%		73.59%		**86.59%**	
Bilan (%)	99.30	98.58	100%	99.19	100%	99.33	100%	99.23	100%

Table N2: Fatty acids composition for the isomerized oil at different times in assay #2

Fatty Acids	Linseed Oil	ISOMERISATION: Assay #2							
		Propylene glycol		Water					
				T = 170C	t = 1h	T = 170C	t = 2h	T = 170C	t = 3h
Saturated									
C16:0	5.40	5.53		5.31		5.17		5.45	
C18:0	4.13	4.26		4.17		3.21		4.18	
Total Saturated	9.53	9.79		9.48		8.38		9.63	
Monoenes									
C18:1	19.77	21.19		20.73		18.43		21.06	
Dienes									
C18:2 9c, 11t	0.00	5.59		0.48		0.89		1.34	
C18:2 9c, 12c	16.47	5.36		15.50		14.58		13.82	
C18:2 10t, 12c	0.00	5.60		0.71		1.12		1.68	
Total C18:2	16.47	16.55		16.69		16.59		16.84	
Isomerisation C18:2 (conjugated / total)	0.00	**67.61%**		7.13%		12.12%		**17.93%**	
Trienes									
C18:3 9c, 11t, 15c et C18:3 9c, 13t, 15c	0.00	**30.94**	60.87%	16.69	76.23%	27.07	72.30%	**28.55**	68.45%
C18:3 9c, 12c, 15c	**53.53**	**0.22**		30.45		18.58		**10.00**	
C18:2 11, 13 cyclohexadiene	0.00	8.32	16.37%	1.79	8.18%	4.94	13.19%	6.47	15.51%
C18:3 conjugated	0.00	11.57	22.76%	3.41	15.59%	5.43	14.50%	6.69	16.04%
Total	53.53	51.05		52.34		56.02		51.71	
Isomerisation C18:3 (conjugated/ total)	0.00	**99.57%**		41.83%		66.83%		**80.66%**	
Bilan (%)	99.30	98.58	100%	99.24	100%	99.42	100%	99.24	100%

Table N3: Fatty acids composition for the isomerized oil at different times in assay #3

Fatty Acids	Linseed Oil	ISOMERISATION: Assay #3							
		Propylene glycol		Volume Water = 0.5; NaOH = 1					
				T = 170C	t = 1h	T = 170C	t = 2h	T = 170C	t = 3h
Saturated									
C16:0	5.40	5.53		5.32		5.45		5.41	
C18:0	4.13	4.26		4.08		4.2		4.03	
Total Saturated	9.53	9.79		9.40		9.65		9.44	
Monoenes									
C18:1	19.77	21.19		20.65		21.13		20.79	
Dienes									
C18:2 9c, 11t	0.00	5.59		0.35		0.68		1.03	
C18:2 9c, 12c	16.47	5.36		15.76		15.25		14.42	
C18:2 10t, 12c	0.00	5.60		0.67		1.04		1.47	
total C18:2	16.47	16.55		16.78		16.97		16.92	
isomerisation C18:2 (conjugated / total)	0.00	**67.61%**		**6.08%**		**10.14%**		**14.78%**	
Trienes									
C18:3 9c, 11t, 15c et C18:3 9c, 13t, 15c	0.00	**30.94**	60.87%	**12.92**	75.73%	**19.44**	71.93%	**24.26**	68.69%
C18:3 9c, 12c, 15c	53.53	**0.22**		35.48		24.79		**17.04**	
C18:2 11, 13 cyclohexadiene	0.00	8.32	16.37%	1.19	6.98%	3.15	11.66%	5.10	14.44%
C18:3 conjugated	0.00	11.57	22.76%	2.95	17.29%	4.44	16.42%	5.96	16.87%
Total	53.53	51.05		52.54		51.82		52.36	
Isomerisation C18:3 (conjugated / total)	0.00	**99.57%**		**32.47%**		**52.16%**		**67.46%**	
Bilan (%)	99.30	98.58	100%	99.37	100%	99.57	100%	99.51	100%

Table N4: Fatty acids composition for the isomerized oil at different times in assay #4

Fatty Acids	Linseed Oil	ISOMERISATION: Assay #4									
		Propylène glycol		Volume Water 1; NaOH 1.5							
				180	t = 0,5h	T = 180C	t = 1h	T = 180C	t = 1,5h	T = 180C	t = 2h
Saturated											
C16:0	5.40	5.53		5.4		5.37		5.43		5.32	
C18:0	4.13	4.26		4.1		4.11		4.21		4.08	
Total Saturated	9.53	9.79	0.00	9.50		9.48		9.64		9.40	
Monoenes											
C18:1	19.77	21.19		20.65		20.92		21.15		20.99	
Dienes											
C18:2 9c, 11t	0.00	5.59		0.94		1.59		2.50		2.99	
C18:2 9c, 12c	16.47	5.36		14.39		13.21		11.23		10.01	
C18:2 10t, 12c	0.00	5.60		1.31		1.97		2.98		3.59	
Total C18:2	16.47	16.55		16.64		16.77		16.71		16.59	
Isomerisation C18:2 (conjugated / total)	**0.00**	**67.61%**		**13.52%**		**21.23%**		**32.79%**		**39.66%**	
Trienes											
C18:3 9c, 11t, 15c et C18:3 9c, 13t, 15c	0.00	**30.94**	60.87%	**25.36**	72.96%	**29.91**	67.36%	**31.49**	63.59%	**31.24**	61.46%
C18:3 9c, 12c, 15c	**53.53**	**0.22**		18.07		7.80		**2.10**		**0.98**	
C18:2 11, 13 cyclohexadiene	0.00	8.32	16.37%	4.54	13.06%	7.35	16.55%	9.02	18.21%	9.60	18.89%
C18:3 conjugés	0.00	11.57	22.76%	4.86	13.98%	7.14	16.08%	9.01	18.19%	9.99	19.65%
Total	53.53	51.05		52.83		52.20		51.62		51.81	
Isomérisation C18:3 (conjugated / total)	**0.00**	**99.57%**		**65.80%**		**85.06%**		**95.93%**		**98.11%**	
Bilan (%)	99.30	98.58	100%	99.62	100%	99.37	100%	99.12	100%	98.79	100%

Table N5: Fatty acids composition for the isomerized oil at different times in assay #5

Fatty Acids	Linseed Oil	ISOMERISATION : Assay # 5									
		Propylene glycol		1,6 x oil mass							
				180	t = 0,5h	T = 180C	t = 1h	T = 180C	t = 1,5h	T = 180C	t = 2h
Saturated											
16:0	5.40	5.53		5.32		5.37		5.27		5.45	
18:0	4.13	4.26		4.09		4.14		4.15		4.19	
Total Saturated	9.53	9.79		9.41		9.51		9.42		9.64	
Monoenes											
18:01	19.77	21.19		20.58		20.83		20.9		21.15	
Dienes											
C18:2 9c, 11t	0.00	5.59		0.40		0.80		1.26		1.63	
C18:2 9c, 12c	16.47	5.36		15.59		14.81		13.77		12.76	
C18:2 10t, 12c	0.00	5.60		0.68		1.20		1.81		2.30	
Total C18:2	16.47	16.55		16.67		16.81		16.84		16.69	
Isomerisation C18:2 (conjugated / total)	**0.00**	**67.61%**		**6.48%**		**11.90%**		**18.23%**		**23.55%**	
Trienes											
C18:3 9c, 11t, 15c et C18:3 9c, 13t, 15c	0.00	**30.94**	60.87%	**11.79**	77.11%	**18.71**	69.58%	**23.75**	65.21%	**26.65**	62.54%
C18:3 9c, 12c, 15c	**53.53**	**0.22**		36.77		25.23		**15.57**		**9.05**	
C18:2 11, 13 cyclohexadiene	0.00	8.32	16.37%	0.58	3.79%	3.25	12.09%	5.34	14.66%	6.78	15.91%
C18:3 conjugated	0.00	11.57	22.76%	2.92	19.10%	4.93	18.33%	7.33	20.13%	9.18	21.54%
Total	53.53	51.05		52.06		52.12		51.99		51.66	
Isomérisation C18:3 (conjugated/ total)	**0.00**	**99.57%**		**29.37%**		**51.59%**		**70.05%**		**82.48%**	
Bilan (%)	99.30	98.58	100%	98.72	100%	99.27	100%	99.15	100%	99.14	100%

Table N7: Fatty acids composition for the isomerized oil at different times in assay #7

Fatty Acids	Linseed Oil	ISOMERISATION: Essay # 7									
		Propylene glycol		Mass: Water 2.6; NaOH 0.50 (Oil = 1)							
				180	t = 1 h	T = 180C	t = 2 h	T = 180C	t = 3 h	T = 180C	t = 4 h
Saturated											
16:0	5.40	5.53		5.29		5.3		5.38		5.37	
18:0	4.13	4.26		4.07		4.13		4.12		4.11	
Total Saturated	9.53	9.79		9.36		9.43		9.50		9.48	
Monoenes											
18:01	19.77	21.19		20.64		20.78		20.98		20.13	
Dienes											
C18:2 9c, 11t	0.00	5.59		0.51		0.93		1.36		1.82	
C18:2 9c, 12c	16.47	5.36		15.49		14.38		13.63		12.68	
C18:2 10t, 12c	0.00	5.60		0.78		1.30		1.82		2.40	
Total C18:2	16.47	16.55		16.78		16.61		16.81		16.90	
Isomerisation C18:2 (conjugated/ total)	**0.00**	**67.61%**		**7.69%**		**13.43%**		**18.92%**		**24.97%**	
Trienes											
C18:3 9c, 11t, 15c et C18:3 9c, 13t, 15c	0.00	**30.94**	60.87%	**14.87**	75.91%	**22.61**	68.58%	**26.51**	67.52%	**28.19**	63.51%
C18:3 9c, 12c, 15c	**53.53**	**0.22**		32.98		19.55		**12.31**		**7.62**	
C18:2 11, 13 cyclohexadiene	0.00	8.32	16.37%	1.79	9.14%	4.17	12.65%	5.98	15.23%	6.94	15.63%
C18:3 conjugated	0.00	11.57	22.76%	2.93	14.96%	6.19	18.77%	6.77	17.24%	9.26	20.86%
Total	53.53	51.05		52.57		52.52		51.57		52.01	
Isomerisation C18:3 (conjugated/ total)	**0.00**	**99.57%**		**37.26%**		**62.78%**		**76.13%**		**85.35%**	
Bilan (%)	99.30	98.58	100%	99.35	100%	99.34	100%	98.86	100%	98.52	100%

Table N8: Fatty acids composition for the isomerized oil in assay #8

Fatty Acids	Linseed Oil	ISOMERISATION: Assay # 8			
		Propylene glycol		Water	
				T=180C	t= 4h
Saturated					
16:0	5.40	5.53		5.47	
18:0	4.13	4.26		4.11	
Total Saturated	9.53	9.79		9.58	
Monoenes					
18:1	19.77	21.19		21.14	
Dienes					
C18:2 9c, 11t	0.00	5.59		1.60	
C18:2 9c, 12c	16.47	5.36		13.23	
C18:2 10t, 12c	0.00	5.60		2.09	
Total C18:2	16.47	16.55		16.92	
Isomerisation C18:2 (conjugated/ total)	0.00	**67.61%**		**21.81%**	
Trienes					
C18:3 9c, 11t, 15c et C18:3 9c, 13t, 15c	0.00	**30.94**	60.87%	**27.97**	66.15%
C18:3 9c, 12c, 15c	**53.53**	**0.22**		9.43	
C18:2 11, 13 cyclohexadiene	0.00	8.32	16.37%	6.47	15.30%
C18:3 conjugated	0.00	11.57	22.76%	7.84	18.54%
Total	53.53	51.05		51.71	
Isomerisation C18:3 (conjugated / total)	0.00	**99.57%**		**81.76%**	
Bilan (%)	99.30	98.58	100%	99.35	100%

Table N9: Fatty acids composition for the isomerized oil in assay #9

	ISOMERISATION: Assay # 9					
	Linseed Oil			*Plukenetia volubilis* Oil		
Fatty Acids	**Initial**	**Isomerisation (Propylene glycol)**		**Initial**	**Isomerisation (Water)**	
Saturated						
C16:0	5.40	5.53		3.74	4.19	
C18:0	4.13	4.26		2.7	3.06	
Total Saturated	9.53	9.79		6.44	7.25	
Monoenes						
C18:1	19.77	21.19		8.93	9.73	
Dienes						
C18:2 9c, 11t		5.59			11.61	
C18:2 9c, 12c	16.47	5.36		31.96	6.86	
C18:2 10t, 12c		5.60			12.43	
Total C18:2	16.47	16.55			30.90	
Isomerisation C18:2 (conjugated/ total)		67.61%			77.80%	
Trienes						
C18:3 9c, 11t, 15c C18:3 9c, 13t, 15c		30.94	60.87%		30.08	60.08%
C18:3 9c, 12c, 15c	53.53	0.22		51.82	0.38	
C18:2 11, 13 cyclohexadiene		8.32	16.37%		7.58	15.14%
C18:3 conjugated		11.57	22.76%		12.41	24.79%
Total	53.53	51.05			50.45	
Isomerisation C18:3 (conjugated/ total)		99.57%			99.25%	
Bilan (%)	99.30	98.58	100.00%	105.59	98	100.00%

Table N10: Summary of isomerisation conditions for different assays

	IN												OUT*			
	Reagents							Reaction Conditions					CLnA™	CLA	Cyclic	Saturated
Assay #	Linseed Oil		Propylene Glycol		NaOH		Total	Temperature	Sample Time				C18:3 9c, 11t, 15c	C18:2 9c, 11t	C18:2 11, 13	
	(g)	(%)	(g)	(%)	(g)	(%)	(g)	(°C)	(h)				C18:3 9c, 13t, 15c	C18:2 10t, 12c	cyclohexadiène	
0	712	8.0%	7,778	87.7%	378	4.3%	8,868	160.00	2.0				30.94	11.19	8.32	9.79
Assay #	Linseed Oil		Water		NaOH		Total	Temperature	Sample Time							
	(g)	(%)	(g)	(%)	(g)	(%)	(g)	(°C)	(h)							
1	712	8.0%	7,778	87.7%	378	4.3%	8,868	180	1,0	1,5	2,0		29.64	3.72	7.40	9.84
2	1428	8.0%	15,794	88.3%	666	3.7%	17,888	170	1,0	2,0	3,0		28.55	3.02	6.47	9,63
3	2804	14.2%	15,556	79.0%	1,324	6.7%	19,684	170	1,0	2,0	3,0		24.26	2.05	5.10	9,44
4	1408	7.8%	15,724	86.8%	974	5.4%	18,106	180	0,5	1,0	1,5	2,0	31.24	6.58	9.60	9,40
5	3520	23.0%	10,904	71.3%	866	5.7%	15,290	180	0,5	1,0	1,5	2,0	26.65	3.93	6.78	9,64
7	2886	18.2%	12264	77.4%	686	4.3%	15,836	180	1,0	2,0	3,0	4,0	28.19	4.22	6.94	9,48
8	2886	18.2%	12292	77.5%	686	4.3%	15,864	180	4,0				27.97	3.69	6.47	9,58
Assay #	*Plukenetia volubilis* Oil		Water		NaOH		Total	Temperature	Sample Time							
	(g)	(%)	(g)	(%)	(g)	(%)	(g)	(°C)	(h)							
9	491	2.9%	15508	90.1%	1220	7.1%	17,219	180	4				30.08	24.04	7.58	7.25

* Results corresponding to the last sample time.

Different purification steps by urea crystallization for CLnA™ compositions

Step			Isomerisation	U1: Urea 1		U2: Urea 2		U3: Urea 3		U4: Urea 4		U5: Urea 5	
From step				over isomerisation		over U1L		over U2L		over U3S		over U4S	
PART (L=liquid; S=solid)				L	S	L	S	L	S	L	S*	L	S*
Current Name	Configuration	# C											
Palmitic		16:0	5.61	0.40	12.61	0.00	0.56	0	0.39	0.1	0.42	0	0.49
Stearic		18:0	4.35	0.06	9.27	0.02	0.24	0.05	0.22	0.06	0.25	0	0.32
Oleic	*cis*-9	18:1	20.86	17.35	25.98	0.69	20.01	2.01	3.25	1.1	3.64	0.38	3.76
cis Vaccenic	*cis*-11	18:1	0.77	0.74	0.86	0.00	0.82	0	0	0	0	0	0
	trans-7,cis-9	18:2											
7,11 - cyclic CLA		18:2											
	cis-8,trans-10	18:2											
	cis-8,cis-10	18:2											
	trans-8,cis-10	18:2											
	cis-9,cis-11	18:2											
Rumenic	***cis-9,trans-11***	**18:2**	**5.43**	**6.41**	4.17	0.47	8.76	0.17	1.4	0.62	1.5	0	1.33
	trans-9,cis-11	18:2											
Linoleic	*cis-9,cis-12*	18:2	5.89	7.41	3.49	7.18	8.33	2.01	9.47	7.42	10.57		9.9
	cis-10,cis-12	18:2											
	cis-10,trans-12	18:2											
	trans-10,cis-12	**18:2**	**5.26**	**6.92**	3.71	1.78	9.26	0.45	3.14	1.63	3.49	0.23	2.95
	cis-11,cis-13	18:2											
	cis-11,trans-13	18:2											
Conjugated Isomers	*trans, trans*	18:2											
11,13 - cyclic CLA		18:2	8.15	11.91	4.04	38.77	0.95	67.75	4.41	18.42	0.42	68.2	0.49
Gamma Rumelenic Acid (φ-CLNA)	*cis-6,trans-8,cis-12*	18:3											
	cis-6,trans-10,cis-12	18:3											
Gamma Linolenic	*cis-6,cis-9,cis-12*	18:3											
	trans-7,cis-9,trans-11	18:3											
Alpha Rumelenic Acid (α-CLNA)	***cis-9,trans-11,cis-15 and cis-9,trans-13,cis-15***	**18:3**	**31.23**	**39.96**	17.83	45.40	41.46	19.17	72.34	62.61	75.35	14.09	75.08
Alpha Linolenic	*cis-9,cis-12,cis-15*	18:3	0.76	0.42	0.39	0.61	0.40	0.31	0.54	0.69	0.62	0.25	0.83
Others Conjugated Isomers of Alpha linolenic	10,12,14 9,11,13[a]	18:3	10.95	7.70	16.57	4.93	8.34	6.53	4.29	7.21	3.51	14.75	4.42

[a]Different full conjugated C18:3 isomers
*Final product is combination of U4S and U5S

New conjugated linolenic acids and methods for commercial preparation and purification

FIELD OF THE INVENTION

This invention relates to a process for preparation and purification of fatty acids which are homologues of conjugated linoleic acids, from materials rich in alpha or gamma linolenic acids. The reaction transforms approximately over ~~one~~ two third of α-linolenic acid (9Z,12Z,15Z-octadecatrienoic acid) into 9Z,11E,15Z-octadecatrienoic acid and 9Z,13E,15Z-octadecatrienoic acid. Enrichment up to and over 40% is readily performed with urea crystallization. Moreover, the product can be produced in over 90% purity by simple preparative liquid chromatography. The reaction is unique in that the reaction produces the abovementioned conjugated trienoic ~~acid~~ acids with a high selectivity, in a short time period and in relatively mild conditions. The reaction also transforms gamma-linolenic acid (6Z,99Z,12Z-octadecatrienoic acid) into 6Z,8E,15Z-octadeccatrienoic acid and 6Z,10E,12Z-octadecatrienoic. In all cases, geometrical isomers and fully conjugated isomers are also produced.

BACKGROUND OF THE INVENTION

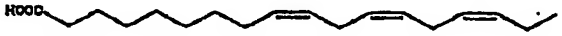
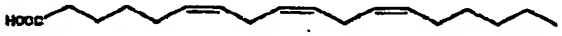
Processes for the conjugation of the double bonds of polyunsaturated unconjugated fatty acids have found their main application in the field of paints and varnishes. Oils comprised of triglycerides of conjugated fatty acids are known as drying oils. Drying oils have value because of their ability to polymerize or "dry" after they have been applied to a surface to form tough, adherent and abrasion resistant films. Tung oil is an example of a naturally occurring oil containing significant levels of fully conjugated fatty acids. Because tung oil is expensive for many industrial applications, research was directed towards finding substitutes.

In the 1930's, it was found that conjugated fatty acids were present in oil products subjected to prolonged saponification, as originally described by Moore, J. Biochem., 31: 142 (1937). This

finding led to the development of several alkali isomerization processes for the production of conjugated fatty acids from various sources of polyunsaturated fatty acids.

The positioning of the double bonds in the hydrocarbon chain is typically not in a conjugated, i.e., alternating double bond-single bond-double bond, manner. For example, α-linolenic acid is an eighteen carbon acid with three double bonds (18:3) at carbons 9, 12 and 15 in which all three double bonds have in the *cis* configuration, i.e., 9Z,12Z,15Z-C18:3 acid, γ-Linolenic acid is 6Z,9Z,12Z-C18:3 acid. Linoleic acid is 9Z,12Z-C18:2 acid (see TABLE 1).

TABLE 1

Nu	Fatty Acid	Trivial Name	Structure
1	9Z,12Z,15Z-C18:3	α-Linolenic Acid	
2	6Z,9Z,12Z-C18:3	γ-Linolenic Acid	
3	9Z,12Z-C18:2	Linoleic Acid	

Migration of double bonds (e.g., leading to conjugation) gives rise to many positional and geometric (i.e., *cis-trans*) isomers.

Conjugated double bonds means two or more double bonds which alternate in an unsaturated compound as in 1,3 butadiene. The hydrogen atoms are on the same side of the molecule in the case of *cis* structure. The hydrogen atoms are on opposite sides of the molecule in the case of *trans* structure.

Conjugated linoleic acid (CLA) is a general term used to name positional and geometric isomers of linoleic acid. Linoleic acid is a straight chain carboxylic acid having double bonds between the carbons 9 and 10, and between carbons 12 and 13. For example, one CLA positional isomer has double bonds between carbons 9 and 10 and carbons 11 and 12 (i.e, 9Z,11E-C18:2 acid); another has double bonds between carbons 10 and 11 and carbons 12 and 13 (i.e., 10E,12Z-C18:2 acid), each with several possible *cis* and *trans* isomers.

TABLE 2

Nu	Fatty Acid	Trivial Name	Structure
	9Z,11E-C18:2	Rumenic Acid	
	10E,12Z-C18:2	none	

The 9Z,11E-C18:2 isomer has been shown to be the first intermediate produced in the biohydrogenation process of linoleic acid by the anaerobic rumen bacterium *Butyrvibrio fibrisolvens*. This reaction is catalyzed by the enzyme ~~linoleate~~ Δ11 isomerase which converts the *cis*-12 double bond of linoleic acid into a *trans*-11 double bond. (C. R. Kepler et al., 241 J. Biol. Chem. (1966) 1350). It has also been found that the normal intestinal flora of rats can also convert linoleic acid to the 9Z,11E-C18:2 acid isomer. The reaction does not, however, take place in animals lacking the required bacteria. Therefore, CLA is largely a product of microbial metabolism in the digestive tract of primarily ruminants, but to a lesser extent in other mammals and birds.

The free, naturally occurring conjugated linoleic acids (CLA) have been previously isolated from fried meats and described as anticarcinogens by Y. L Ha, N K. Grimm and M. W. Pariza, in Carcinogenesis, Vol. 8, No. 12, pp. 1881-1887 (1987). Since then, they have been found in some processed cheese products (Y. L. Ha, N. K. Grimm and M. W. Pariza, in J. Agric. Food Chem., Vol. 37, No. 1, pp. 75-81 (1987)). Cook et al. in U.S. Pat. No. 5,554,646 disclose animal feeds containing CLA, or its non-toxic derivatives, e.g., such as sodium and potassium salts of CLA, as an additive in combination with conventional animal feeds or human foods. CLA makes for leaner animal mass.

The biological activity associated with CLAs is diverse and complex (Pariza et al. in Prog. Lipid Research., Vol 40, pp. 283-298).

Anti-carcinogenic properties have been well documented, as well as stimulation of the immune system. Administration of CLA inhibits rat mammary tumorogenesis, as demonstrated by Ha et

al., Cancer Res., 52:2035-s (1992). Ha et al., Cancer Res., 50:1097 (1990), reported similar results in a mouse forestomach neoplasia model. CLA has also been identified as a strong cytotoxic agent against target human melanoma, colorectal and breast cancer cells in vitro. A recent major review article confirms the conclusions drawn from individual studies (Ip, Am. J. Clin. Nutr. 66(6):1523s (1997)). In *in vitro* tests, CLAs were tested for their effectiveness against the growth of malignant human melanomas, colon and breast cancer cells. In the culture media, there was a significant reduction in the growth of cancer cells treated with CLAs by comparison with control cultures. The mechanism by which CLAs exert anticarcinogenic activity is unknown. In addition, CLAs have a strong antioxidative effect so that, for example, peroxidation of lipids can be inhibited (Atherosclerosis 108, 19-25 (1994)). U.S. Pat. No. 5,914,346 discloses the use of CLA's to enhance natural killer lymphocyte function. U.S. Pat. No. 5,430,066 describes the effect of CLA's in preventing weight loss and anorexia by immune system stimulation.
Although the mechanisms of CLA action are still obscure, there is evidence that some component(s) of the immune system may be involved, at least in vivo. U.S. Pat. No. 5,585,400 (Cook, et al., incorporated herein by reference), discloses a method for attenuating allergic reactions in animals mediated by type I or IgE hypersensitivity by administering a diet containing CLA. CLA in concentrations of about 0.1 to 1.0 percent was also shown to be an effective adjuvant in preserving white blood cells. U.S. Pat. No. 5,674,901 (Cook, et al., incorporated herein by reference), disclosed that oral or parenteral administration of CLA in either free acid or salt form resulted in elevation in CD-4 and CD-8 lymphocyte subpopulations associated with cell mediated immunity. Adverse effects arising from pretreatment with exogenous tumor necrosis factor could be alleviated indirectly by elevation or maintenance of levels of CD-4 and CD-8 cells in animals to which CLA was administered.

CLA's have also been found to exert a profound generalized effect on body composition, in particular, upon redirecting the partitioning of fat and lean tissue mass. U.S. Pat. Nos. 5,554,646 and 6,020,378 disclose the use of CLA's for reducing body fat and increasing lean body mass. U.S. Pat. No. 5,814,663 discloses the use of CLA's to maintain an existing level of body fat or body weight in humans. U.S. Pat. No. 6,034,132 discloses the use of CLA's to reduce body weight and treat obesity in humans. CLA's are also disclosed by U.S. Pat. No. 5,804,210 to maintain or enhance bone mineral content. EP 0 579 901 B relates to the use of CLA for avoiding

loss of weight or for reducing increases in weight or anorexia caused by immunostimulation in human beings or animals. U.S. Pat. No. 5,430,066 (Cook, et al., incorporated herein by reference), describes the effect of CLA in preventing weight loss and anorexia by immune stimulation.

CLA has been found to be an in vitro antioxidant, and in cells, it protects membranes from oxidative attack. In relation to other important dietary antioxidants, it quenches singlet oxygen less effectively than beta.-carotene but more effectively than .alpha.-tocopherol. It appears to act as a chain terminating antioxidant by chain-propagating free radicals (U.S. Pat. No. 6,316,645 (Sih, et al., incorporated herein by reference)).

Skin is subject to deterioration through dermatological disorders, environmental abuse (wind, air conditioning, central heating) or through the normal aging process (chronoaging) which may be accelerated by exposure of skin to sun (photoaging). In recent years the demand for cosmetic compositions and cosmetic methods for improving the appearance and condition of skin has grown enormously. WO 95/13806 discloses the use of a composition comprising zinc salts of 68% (unconjugated) linoleic acid and 10% conjugated isomers of linoleic acid for use in treating skin disorders.

Apart from potential therapeutic and pharmacologic applications of CLA as set forth above, there has been much excitement regarding the use of CLA as a dietary supplement. CLA has been found to exert a profound generalized effect on body composition, in particular redirecting the partitioning of fat and lean tissue mass. U.S. Pat. No. 5,554,646 (Cook, et al., incorporated herein by reference), discloses a method utilizing CLA as a dietary supplement in which pigs, mice, and humans were fed diets containing 0.5 percent CLA. In each species, a significant drop in fat content was observed with a concomitant increase in protein mass. It is interesting that in these animals, increasing the fatty acid content of the diet by addition of CLA resulted in no increase in body weight, but was associated with a redistribution of fat and lean within the body. Another dietary phenomenon of interest is the effect of CLA supplementation on feed conversion. U.S. Pat. No. 5,428,072 (Cook, et al., incorporated herein by reference), provided data showing that incorporation of CLA into animal feed (birds and mammals) increased the efficiency of feed

conversion leading to greater weight gain in the CLA supplemented birds and mammals. The potential beneficial effects of CLA supplementation for food animal growers is apparent.

Another important source of interest in CLA, and one which underscores its early commercial potential, is that it is naturally occurring in foods and feeds consumed by humans and animals alike. In particular, CLA is abundant in products from ruminants. For example, several studies have been conducted in which CLA has been surveyed in various dairy products. Aneja, et al., (J. Dairy Sci., 43: 231 [1990]) observed that processing of milk into yogurt resulted in a concentration of CLA. Shanta, et al. (Food Chem., 47: 257 [1993]) showed that a combined increase in processing temperature and addition of whey increased CLA concentration during preparation of processed cheese. In a separate study, Shanta, et al., J. Food Sci., (60: 695 [1995]) reported that while processing and storage conditions did not appreciably reduce CLA concentrations, they did not observe any increases. In fact, several studies have indicated that seasonal or interanimal variation can account for as much as three fold differences in CLA content of cows milk (Parodi, et al., J. Dairy Sci., 60: 1550 [1977]). Also, dietary factors have been implicated in CLA content variation (Chin, et al., J. Food Comp. Anal., 5: 185 [1992]). Because of this variation in CLA content in natural sources, ingestion of prescribed amounts of various foods will not guarantee that the individual or animal will receive the optimum doses to ensure achieving the desired nutritive effect.

Economical conjugated fatty acid production in commercial quantities for use in domestic food animal feeds is a desirable objective in light of the nutritional benefits realized on a laboratory scale. Preferably, the conjugated fatty acid is produced directly from a source of raw vegetable oil and not from expensive purified linoleic acid. Further, the process must avoid cost generating superfluous steps, and yet result in a safe and wholesome product palatable to animals.
All the useful methodologies for preparation of conjugated linoleic acid (CLA) were recently reviewed by Adlof (In:Yurawecz *et al.* (Ed), Advances in Conjugated Linoleic Acid Research, volume 1, AOCS Press, Champaign, Il, pp 21-38 [1999]).

The usual methodology for conjugation of polyunsaturated fatty acids is alkali-catalyzed isomerization. This reaction may be performed using different bases such as hydroxides or

alkoxides in solution in appropriate alcoholic reagents. This reaction was developed in the 1950's for spectrophotometric estimation of polyunsaturated fatty acids in fats and oils [AOCS official method Cd 7-58; JAOCS 30:352 (1953)].

In alkali isomerization the fatty acids are exposed to heat, pressure and a metal hydroxide or oxide in nonaqueous or aqueous environments, resulting in the formation of conjugated isomers. Other methods have been described which utilize metal catalysts, which is not as efficient in the production of conjugated double bonds. It was found that isomerization could be achieved more rapidly in the presence of higher molecular weight solvent. Kass, et al., J. Am. Chem. Soc., 61: 4829 (1939) and U.S. Pat. No. 2,487,890 (1950) showed that replacement of ethanol with ethylene glycol resulted in both an increase in conjugation in less time. U.S. Pat. No. 2,350,583 and British Patent No. 558,881 (1944) achieved conjugation by reacting fatty acid soaps of an oil with an excess of aqueous alkali at 200-230 degrees C. and increased pressure.

Dehydration of methyl ricinoleate (methyl 12-hydroxy-*cis*-9-octadecenoate) (Gunstone and Said, Chem. Phys. Lipids 7, 121 [1971]; Berdeaux et al., JAOCS 74, 1011 [1997] give 9Z,11E-C18:2 isomer as a major product. U.S. Pat. Nos. 5,898,074 disclosed a synthesis process for producing this fatty acid at room temperature in high yield. The tosylate or the mesylate of the methyl ester of ricinoleic acid is formed with tosyl chloride or mesyl chloride in a pyridine solvent or in acetonitrile and triethyl amine. The obtained tosylate or mesylate is reacted with diazabicyclo-undecene in a polar, non-hydoxylic solvent of acetonitrile to form the preferred isomer of 9c,11t-18:2 methyl ester in high yield. U.S. Pat. Nos. 6,160,141 disclosed a synthesis process for producing conjugated eicosanoid fatty acid from methyl lesquerolate (methyl 14-hydroxy-*cis*-11-octadecenoate) at room temperature in high yield using the same principle.

Among the processes known to effect isomerization without utilizing an aqueous alkali system, is a nickel-carbon catalytic method, as described by Radlove, et al., Ind. Eng. Chem.38: 997 (1946). A variation of this method utilizes platinum or palladium-carbon as catalysts. Conjugated acids may also be obtained from α-hydroxy allylic unsaturated fatty acid using acid catalyzed reduction (Yurawecz et al., JAOCS 70, 1093 [1993]), and partial hydrogenation of conjugated acetylenic acid such as santalbic (11E-octadec-9-ynoic) acid using Lindlar's catalyst could also be used but are limited by natural sources of such fatty acid. Another approach uses strong

organic bases such as butyllithium It has been applied to both the conjugation of linoleic acid and partial and full conjugation of alpha-linolenic acid ((U.S. Pat. No. 6,316,645 (Sih, et al., incorporated herein by reference)).

Natural fully conjugated linolenic acids have been found at high content levels in some seed oils (Hopkins, In:Gunstone, F.D. (Ed), Topics in Lipid Chemistry, volume 3, ELEK Science, London, pp 37-87 [1972]). For example, Takagi and Itabashi (Lipids 16, 546 [1981]) reported calendic acid (8E,10E,12Z-C18:3 acid, 62.2%) in pot marigold seed oil, punicic acid (9Z,11E,13Z-C18:3 acid, 83.0%) in pomegranate seed oil, α-eleostearic acid (9Z,11E,13E-C18:3 acid) in tung (67.7%) and bitter gourd (56.2%) seed oils, and catalpic acid (9E,11E,13Z-C18:3 acid, 42.3%) in catalpa seed oil, respectively.

An octadecatrienoic acid isomer whose structure has been tentatively defined as 9Z,11E,15Z-C18:3 acid, is believed to be the first intermediate in the biohydrogenation process of α-linolenic acid by the anaerobic rumen bacterium *Butyrvibrio fibrisolvens* (C. R. Kepler and S. B. Tove 242 J. Biol. Chem. (1967) 5686).

SUMMARY OF THE INVENTION

The present invention describes a process for preparation and purification of fatty acids which are homologues of conjugated linoleic acids from natural and/or synthetic materials richin alpha or gamma linolenic acids or both. In a preferred embodiment the reaction ~~produces~~ transforms approximately over ~~one~~ two thirds of alpha linolenic acid (9Z,12Z,15Z-C18:3 acid) from a natural source such as linseed oil ~~from~~ into 9Z,11E,15Z and 9Z,13E,15Z C18:3 acids, producing a mixture containing approximately 30% of the said conjugated linolenic acids. In a second embodiment, enrichment up to and over 40% is readily performed with urea crystallization. Moreover, the product can be produced in over 90% purity by simple preparative liquid chromatography. In another embodiment, the products obtained include free fatty acids, and derivatives thereof, including, but not limited to esters, amides, salts, fatty alcohols. The reaction

is unique in that the reaction produces the abovementioned conjugated trienoic acid with a high selectivity, in a short time period and in relatively mild conditions.

BRIEF DESCRIPTION OF DRAWINGS

Fig. 1 presents mass spectra of products resulting of the isomerization process of alpha-linolenic acid (9Z,12Z,15Z-C18:3 acid), as 4,4-dimethyloxazoline derivatives: A, 9Z,11E,15Z and 9Z,13E,15Z-C18:3; B, 9,11,13-C18:3, C, 10E,12Z,14E-C18:3 and D, 11,13-CCLA (9-(6-propyl-cyclohexa-2,4-dienyl)-nonanoic acid);

Fig. 2 present the thermal mechanism leading to the formation of 11,13-CCLA [9-(6-propyl-cyclohexa-2,4-dienyl)-nonanoic acid (Fig 1-D)] from 10E,12Z,14E-C18:3 acid.

Fig. 3 present gas liquid chromatograms of fatty acid methyl esters obtained after methylation of linseed oil (A), conjugated linseed oil (B) liquid phase from urea crystallization (C), reversed-phase liquid chromatography fraction containing about 97 % of a mixture of 9Z,11E,15Z and 9Z,13E,15Z-C18:3 acids (D), argentation liquid chromatography fraction containing about 99+ % of a mixture of 9Z,11E,15Z and 9Z,13E,15Z -C18:3 acids (E);

Fig. 4 presents the gas liquid chromatogram of the fatty acid methyl esters obtained after methylation of partially conjugated evening primrose oil.

DETAILED DESCRIPTION OF THE INVENTION

The oils and fats, alone or as mixtures, containing alpha-linolenic acid may include but are not limited to arnebia, basil, candelnut, flax (linseed), linola, gold of pleasure, hemp, mustard, perilla, soybean, canola, walnut, chia, crambe, echium, hop, kiwi, pumkin, black currant and purslane seed oils, or any other oil, wax, ester or amide that is rich in linolenic acid.

The oils and fats, alone or as mixtures, containing gamma-linolenic acid may include but are not limited to borage, evening primrose and black currant seed oils, or any other oil, wax, ester or amide that is rich in linolenic acid.

The disclosed process converts double bonds of α- and γ-linolenic acid isomers into partly and/or fully conjugated systems as well as into cyclic fatty acid isomers. The process which can be performed both in batch and continuous modes, involves blending one or a mixture of vegetable oils with various concentration of alpha or gamma linoleic acids or both or partial glycerides of such oils, or partially purified or concentrated isomers with 0.5 to 10 moles of base such as sodium hydroxide, sodium alkoxylate, sodium metal, potassium hydroxide, potassium alkoxylate, potassium metal, and strong base resins. The reaction proceeds at temperatures from 20°C up to 280°C in the solvent, selected from commercial polyols such as propylene glycol, glycerol and ethylene glycol, for periods of 30 sec to 18 hours, depending on the base and/or the temperature and/or solvent, and/or substrate and/or a desire expected conversion rate. After cooling, if required, to 20-80°C, acid is added to the reaction mixture to neutralize the soaps and/or remaining base in the reactor. It is preferred to bring the pH of the contents of the reactor to pH4 or less through the addition of either a mineral or organic acid. Acids that may be used include, but are not limited to, hydrochloric acid, sulfuric acid, phosphoric acid and citric acid. The solvent phase (polyol + water) is withdrawn and the remaining fatty acid rich phase can be washed with water and/or saline solutions of variable concentration such as sodium chloride (5%w/w) to remove traces of acids used for acidification of the reaction mixture. Remaining water can be removed by usual means (i.e. centrifugation, vacuum, distillation or drying agents). As described in example 1, the concentration of 9Z,11E,15Z and 9Z,13E,15Z -C18:3 acid in the product is approximately 33%. This product, as such or converted into derivatives, can be used in nutrition, cosmetic, nutraceutical, biological and/or animal feed applications.

Isomer composition of the formed fatty acid was determined by gas-liquid chromatography coupled with a mass-spectrometer (GC-MS) of their 4,4-dimethyloxazoline (DMOX) derivatives. The use of derivatives is a necessary step prior to structural determination of fatty acid by GC-MS because mass spectra of fatty acid methyl ester, the usual derivatives for gas-liquid chromatography analysis, are devoid of sufficient information for identification of structural

isomers. This is mainly due to the high sensitivity of the carboxyl group to fragmentation and to double bond migration (Christie, W.W., Gas Chromatography–Mass Spectrometry Methods for Structural Analysis of Fatty Acids, Lipids 33:343–353 (1998).). However, stabilization of the carboxyl group by the formation of a derivative containing a nitrogen atom results in mass spectra that allow structural determination for most fatty acids. Indeed, these fatty acids derivatives provide diagnostic fragments that allow accurate structure determination. The derivatives were submitted to GC-MS with a Hewlett Packard 5890 Series II plus gas chromatograph attached to an Agilent model 5973N MS Engine. The latter was used in the electron impact mode at 70 eV with a source temperature of 230.degree. C. The GC was fitted with split injection. For DMOX derivatives an open tubular capillary column coated with BPX-70 (60 m.times.0.25 mm, 0.25 μm film; SGE, Melbourne, Australia) was used. After holding the temperature at 60.degree. C. for 1 min, the oven temperature was increased by temperature-programming at 20.degree. C./min to 170.degree. C where it was held for 30 min., then at 5.degree. C./min to 210.degree. C. where it was held for 30 min. Helium was the carrier gas at a constant flow-rate of 1 mL/min, maintained by electronic pressure control.

Mass spectrum of conjugated products of 9Z,12Z,15Z-C18:3 acid obtain by conjugation of linseed oil were presented in FIG. 1.

Structural formula and mass spectrum of the DMOX derivatives of the 9Z,11E,15Z-C18:3 acid are illustrated in FIG. 1A. The DMOX has a molecular ion at m/z=331, confirming the octadecatrienoic acid structure. The ion at m/z=262 confirms the location of the 11,15-double bond system (by extrapolation from the known 5,9-isomer (Berdeaux and Wolff, J. Am. Oil Chem. Soc., 73: 1323-1326 (1996)), similarly molecular ion at m/z=236 confirms the location of the 9,13-double bond system, and gaps of 12 a.m.u. between m/z=208 and 196, and 288 and 276 verify the location of double bonds in positions 9 and 15, respectively. Mass spectrometry does not confirm the geometry of the double bonds, but they have been determined according to Nichols et al. (J. Am. Chem. Soc, 73:247-252 (1951)) based on the Ingold theory on the prototropic shift mechanism (Ingold, J. Chem. Soc, 1477 (1926)).

Structural formula and mass spectrum of the DMOX derivatives of the 9,11,13-C18:3 acid are illustrated in FIG. 1B. The DMOX has a molecular ion at m/z=331, confirming the octadecatrienoic acid structure. Gaps of 12 a.m.u. between m/z=208 and 196, and 222 and 234, and 248 and 260 verify the location of double bonds in positions 9 ,11 and 13, respectively. Four different minor isomers of 9,11,13-C18:3 are present in the reaction products. The most abundant is the 9Z,11Z,13E-C18:3 acid isomer which is known as α-eleostearic acid.

Mass spectra of the MTAD adducts of *cis*-9,*trans*-11,*cis*-15 18:3 (A) and *cis*-9, *trans*-13,*cis*-15 18:3 (B) acid methyl esters and presented at FIG 2.

Structural formula and mass spectrum of the DMOX derivatives of the 10E,12Z,14E-C18:3 acid are illustrated in FIG. 1C. The DMOX has a molecular ion at m/z=331, confirming the octadecatrienoic acid structure. Gaps of 12 a.m.u. between m/z=210 and 222, and 236 and 248, and 262 and 274 verify the location of double bonds in positions 10 ,12 and 14, respectively. Mass spectrometry does not confirm the geometry of the double bonds, but they have been determined according to Nichols et al. (J. Am. Chem. Soc, 73:247-252 (1951)) based on the Ingold theory on the prototropic shift mechanism (Ingold, J. Chem. Soc, 1477 (1926)). The 10E,12Z,14E-C18:3 acid isomer is prone to cyclization, thus forming cyclohexadienyl compound (9-(6-propyl-cyclohexa-2,4-dienyl)-nonanoic acid)) by an electrocyclization process presented in FIG. 3.

Structural formula and mass spectrum of the DMOX derivatives of the 11,13-CCLA (9-(6-propyl-cyclohexa-2,4-dienyl)-nonanoic acid) are illustrated in FIG. 1D. The DMOX has a molecular ion at m/z=330 −1, confirming the occurrence of a high stabilized conjugated ion fragment (radical in carbon 10 or 15, stabilized by resonance effect). A distinctive ion at m/z=288 is characteristic for alpha cleavage occurring in cyclic fatty acids (Sébédio at al. J. Am. Oil Chem. Soc., 64: 1324-1333 (1987)). The gap of 78 atomic mass units (a.m.u.) between m/z=288 and 210 is that expected for the cyclohexadienyl group which conjugated double bond system in positions 11 and 13.

Reaction progress was determined by gas-liquid chromatography under appropriate condition as presented in EXAMPLE 1.

Increasing the concentration of, for example 9Z,11E,15Z and 9Z,13E,15Z-C18:3 acids, can be achieved using different methods, alone or in combination. One method makes use of urea complexation. Urea solution, prepared at a temperature ranging from 20 to 90°C in different solvents or mixtures thereof, selected from water, and/or alcohols. Complexation is performed at the same temperature by addition of the product in a molar ratio of 0.5 to 8, and cooling at a temperature range of 30 to -30°C, as required. A mixture of the abovementioned 9Z,11E,15Z and 9Z,13E,15Z -C18:3 acids is isolated in higher concentration after treatment of the liquid phase, obtained after separation from the solid phase, by using conventional means such as filtration or centrifugation. Decomplexation is then carried out by addition of either a diluted organic or mineral acid. Acids that may be used include, but are not limited to, hydrochloric acid, sulfuric acid, phosphoric acid and citric acid. The product is obtained by decantation or liquid-liquid extraction with an organic solvent such as but not limited to hexane, heptane, petroleum ether and ligroin. If required, the organic solvent is eliminated (i.e. evaporated or distilled). A preferred description of the present embodiment is described in example 2.

Another method for raising level of, for example 9Z,11E,15Z and 9Z,13E,15Z -C18:3 acids, either as free acid or derivative (i.e. methyl, ethyl, isopropyl, butyl, phenyl) is liquid chromatography using various convenient stationary phases. One particular is reversed phase liquid chromatography (i.e. ODS) for which eluents may include but are not limited to water, acetonitrile, acetone, methanol, tetrahydrofuran, methyl-tertbutyl ether, and combination thereof. A detailed description of the method is described in example 3. Argentation liquid chromatography may be used to isolate specific isomers from a complex mixture of fatty acid ester or free fatty acid. A detailed description of the methodology applied to a mixture of 9Z,11E,15Z and 9Z,13E,15Z -C18:3 acid isomers is described in example 4.

Still another method for raising the concentration level of, for example a mixture of 9Z,11E,15Z and 9Z,13E,15Z -C18:3 acid, either as free acid or derivative (i.e. methyl, ethyl, isopropyl, butyl, phenyl) is crystallization, either in solvent or mixture thereof, such as, but not limited to, acetone, methanol, pentane, or in absence of solvent (i.e. dry fractionation). A detailed cooling program is

required in order to obtain a more concentrated product. One particular case is that of further crystallization of urea complexes of fatty acids.

EXPERIMENTAL

The following examples are provided in order to demonstrate and further illustrate certain preferred embodiments and aspects of the present invention and are not to be construed as limiting the scope thereof.

In the experimental disclosure which follows, the following abbreviations apply: kg (kilograms); g (grams); mg (milligrams); °C (degrees centigrade); L (liters); mL (milliliters); µL (microliters); m (meters); cm (centimeters); mm (millimeters), µm (micrometers); NaOH (sodium hydroxide), H_2SO_4 (sulfuric acid), NaCl (sodium chloride); 11,13-CCLA (9-(6-propyl-cyclohexa-2,4-dienyl)-nonanoic acid), $AgNO_3$ (silver nitrate).

EXEMPLE 1

Preparation of mixture containing high amount of 9Z,11E,15Z and 9Z,13E,15Z -C18:3 acids by conjugation of linseed oil

To commercial propylene glycol (46.48 kg) were added NaOH (1.94 kg) at room temperature. The resulting mixture was heated at 160°C for 20 min into a 200 L stainless steal reactor under nitrogen atmosphere and with vigorous agitation. Commercial raw linseed oil (4.19 kg) was added under nitrogen atmosphere. The mixture was heated at 160°C for 2 hours under nitrogen atmosphere and with vigorous agitation. After cooling to 80°C, the reaction mixture was directly acidified with aqueous solution of H_2SO_4 (0.06 % w/w, 47.5 kg). After standing 10 min, the top layer was washed with a NaCl aqueous solution (5% w/w, 47.25 kg). The top layer was removed, dried and stored at -80°C under nitrogen.

Fatty acid composition of the resulting product was performed by high resolution gas-chromatography after methylation of a sample (20 mg) using the boron trifluoride method (Metcalfe et al.,). The analytical equipment consisted of an Agilent Technologies GLC 6890 with

auto sampler. The column was a highly polar open tubular capillary type. The following program setting were used (TABLE 3)

TABLE 3

Injection	Split mode 1:50 at 250°C
Detection	Flame Ionization Detector at 250°C
Carrier	Helium at 249.5 KPa at 170°C
Oven Program	60°C for 1 min then 20°C.min^{-1} to 170°C and 170°C throughout for 30 min, then 5°C.min^{-1} 210°C C throughout for 5 min
Column	BPX-70 capillary column, 60 m X 0.25 mm i.d., 0.25 µm film thickness

The obtained chromatogram was given in fig 4 B. The quantitative conversion of alpha-linolenic acid was confirmed and the mixture contain approximately 33 % of 9Z,11E,15Z and 9Z,13E,15Z -C18:3, fatty acid composition of the mixture was given in table 4.

TABLE 4

Fatty Acid	% Before Reaction	% After Reaction
Palmitic	5.40	5.07
Stearic	4.13	3.20
Oleic	19.77	19.27
11Z-C18:1	0.69	0.65
Linoleic	16.47	7.16
alpha-Linolenic	53.54	0.87
9Z,11E-C18:2	0.00	4.89
10E,12Z-C18:2	0.00	4.79
11,13-CCLA	0.00	8.73
9Z,11E,15Z-C18:3	0.00	32.98
9,11,13-C18:3[1]	0.00	3.73
10E,12Z,14E-C18:3	0.00	6.06
10,12,14- C18:3[2]	0.00	1.41

[1]stereochemistry of the double bonds not identified

[2]other stereo isomers of 10,12,14-C18:3 Acid

EXEMPLE 2

Préparation of mixture containing high amount of a mixture of 9Z,11E,15Z and 9Z,13E,15Z - C18:3 acid by conjugation of linseed oil and consecutive urea crystallization

The top layer (3.26 kg) obtained in example 1 was removed and transferred in a 20 L reactor containing a solution of urea (3.26 kg) in aqueous ethanol (95 %, v/v, 13.20 kg) prepared at 60°C under nitrogen condition for 1 hour. Free fatty acids were homogenized and the obtained mixture was cooled at 4°C for 12 h. The liquid phase (17.77 kg) was removed from the solid phase (3.18 kg) by centrifugation and transferred into a 100 L, stainless steal, sight glasses reactor. An aqueous solution of H_2SO_4 (0.1 %, w/w, 49.12 kg) was added to the mixture and the solution was vigorously shaken for 1 min under nitrogen atmosphere. After standing 10 min, the top layer was washed with a NaCl aqueous solution (5% w/w, 47.25 kg). The top layer was removed, dried and stored at -80°C under nitrogen.

The solid phase (3.18 kg) was dissolved in a solution of H_2SO_4 (0.1 %, w/w, 49.12 kg) at 70°C and transferred into a 107 L, stainless steal, sight glasses reactor and the solution was vigorously shaken for 1 min under nitrogen atmosphere. After standing 10 min, the top layer was washed in the same apparatus with a NaCl aqueous solution (5% w/w, 47.25 kg). The top layer was removed, dried and stored at -80°C under nitrogen.

Fatty acid composition of the resulting products was performed by high resolution gas-chromatography after methylation of samples (20 mg) using the boron trifluoride method (Metcalfe et al.,). The used analytical conditions were the same as presented in example 1.

The obtained chromatogram was given in fig 4C and fatty acid composition of the mixture was given in table 5.

TABLE 5

Fatty Acid	% Before Crystallization	% in Liquid Phase	% in Solid Phase
Palmitic	5.07	0.58	15.41
Stearic	3.20	0.04	12.17
Oleic	19.27	17.19	27.88
11Z-C18:1	0.65	0.66	0.84
Linoleic	7.16	8.50	2.60

alpha-Linolenic	0.87	0.79	0.17
9Z,11E-C18:2	4.89	5.86	4.17
10E,12Z-C18:2	4.79	6.21	2.59
11,13-CCLA	8.73	10.61	1.42
9Z,11E,15Z and 9Z,13E,15Z -C18:3	32.98	40.74	10.88
9,11,13-C18:3[1]	3.73	3.54	3.17
10E,12Z,14E-C18:3	6.06	0.73	13.78
10,12,14- C18:3[2]	1.41	1.26	1.72

[1]stereochemistry of the double bonds not identified

[2]other stereo isomers of 10,12,14-C18:3 Acid

EXEMPLE 3

Preparation and purification of a mixture of 9Z,11E,15Z and 9Z,13E,15Z -C18:3 acids by reverse phase liquid chromatography.

Products obtained in example 1 and 2 containing a high level of 9Z,11E,15Z and 9Z,13E,15Z -C18:3 were submitted to a preparative high performance liquid chromatograph fitted with a preparative ODS (octadecylsilyl) column (25 cm X 6.5 cm i.d.). The mobile phase was methanol and water (90:10, v/v, 400 $mL.min^{-1}$). The sample (10 g) was injected at atmospheric pressure and the separation was achieved in 60 min. Collected fractions were analyzed by gas-liquid chromatography as presented in example 1, and a typical gas-chromatogram was presented in fig 4D. The desired compounds eluted in the first partition (partition number = 12) that allow a purification of about 95 %.

EXEMPLE 4

Preparation and purification of 9Z,11E,15Z and 9Z,13E,15Z -C18:3 acids by argentation liquid chromatography

Fatty acid methyl esters prepared from products obtained in example 1 and 2 that containing a high level of a mixture of 9Z,11E,15Z and 9Z,13E,15Z -C18:3 were separated using argentation thin layer chromatography. Silica-gel plates were prepared by immersion in a 5% acetonitrile solution of $AgNO_3$ as described by Destaillats et al. (Lipids 35:1027-1032, (2000)). The developing solvent was the mixture n-hexane/diethyl ether (90:10, v/v). At the end of the

chromatographic runs, the plates were briefly air-dried, lightly sprayed with a solution of 2',7'-dichlorofluorescein, and viewed under ultraviolet light (234 nm). The band at R_f = 0.52 was scraped off and eluted several times with diethyl ether. Complete evaporation of the combined extracts was achieved with a light stream of dry nitrogen. The residues were dissolved in an appropriate volume of n-hexane and analysed by gas-liquid chromatography (purity superior to 98 %) as presented in example 1.

EXEMPLE 5

Preparation of mixture containing 6Z,8E,12Z, 6Z,10E,12Z- and 6Z,9Z,12Z-C18:3 acids by partial conjugation of borage oil

To commercial propylene glycol (96 g) were added NaOH (4.30 g) at room temperature. The resulting mixture was heated at 160°C for 20 min under nitrogen atmosphere and with vigorous agitation. Commercial borage oil (9.35 g) was added under nitrogen atmosphere. The mixture was heated at 160°C for 1 hour under nitrogen atmosphere and with vigorous agitation. After cooling to 80°C, the reaction mixture was directly acidified with aqueous solution of H_2SO_4. After standing 10 min, the top layer was washed with a 5% NaCl aqueous solution (w/w, 47.25 kg),removed, dried and stored at -80°C under nitrogen.

Fatty acid composition of the resulting products was performed by high resolution gas-chromatography after methylation of samples (20 mg) using the boron trifluoride method (Metcalfe et al.,). The used analytical conditions were the same as presented in example 1.

The obtained chromatogram was given in fig 5 and fatty acid composition of the mixture was given in table 6.

TABLE 6

Fatty Acid	% Before Reaction	% After Reaction
Palmitic	10.34	9.55
Stearic	3.36	2.38
Oleic	15.57	13.88
11Z-C18:1	0.57	0.52
Linoleic	39.96	30.11
γ-Linolenic	22.92	5.32
7,11-CCLA	0.00	1.25
9Z,11E-C18:2	0.00	6.66
10E,12Z-C18:2	0.00	6.46
9Z-C20:1	3.69	2.60
6Z,8E,12Z and 6Z,10E,12Z -C18:3	0.00	14.50
9Z-C22:1	2.05	1.22
7E,9Z,11E-C18:3	0.00	1.89

He K, Rimm EB, Merchant A, Rosner BA, Stampfer MJ, Willett WC, Ascherio A (2002) Fish consumption and risk of stroke in men. *JAMA* 288(24):3130-3136.

Igarashi M, Miyazawa T (2000a) Do conjugated eicosapentaenoic acid and conjugated docosahexaenoic acid induce apoptosis via lipid peroxydation in cultured human tumors cells? *Biochem Biophys Res Commun* 270:649-656.

Igarashi M, Miyazawa T (2000b) Newly recognized cytotoxic effect of conjugated trienoic fatty acids on cultured human tumor cells. *Cancer Lett* 148:173-179.

Iso H, Rexrode KM, Stampfer MJ, Manson JE, Colditz GA, Speizer FE, Hennekens CH, Willet WC (2001) Intake of fish and omega-3 fatty acids and risk of stoke in women. *JAMA* 285(3):304-312.

Ip C, Banni S, Angioni E, Carta G, McGinley J, Thompson HJ, Barbano D, Bauman D (1999) Conjugated linoleic acid-enriched butter fat alters mammary gland morphogenesis and reduces cancer risk in rats. *J Nutr* 129(2):2135-2142.

Koba K, Akahoshi A, Yamasaki M, Tanaka K, Yamada K, Iwata T, Kamegai T, Tsutsumi K, Sugano M (2002) Dietary conjugated linolenic acid in relation to CLA differently modifies body fat mass and serum and liver lipid levels in rats. *Lipids* 37:343-350.

Krietchevsky D, Tepper SA, Wright S, TsoP, Czarnecki SK (2000) Influence of conjugated linoleic acid (CLA) on establishment and progression of atherosclerosis in rabbits. *J Am Coll Nutr* 19:472S-477S.

Lee KN, Kritchevsky D, Pariza MW (1994) Conjugated linoleic acid and atherosclerosis in rabbits *Atherosclerosis* 108:19-25.

Liu JR, Chen BQ, Yang YM, Wang XL, Xue YB, Zheng YM, Liu RH. (2002) Effect of apoptosis on gastric adenocarcinoma cell line SGC-7901 induced by cis-9, trans-11-conjugated linoleic acid. *World J Gastroenterol* 8(6):999-1004.

Marchioli R, Schweiger C, Tavazzi L, Valagussa F (2001) Efficacyof n-3 polyunsaturated fatty acids after myocardial infarction: results of GISSI-prevenzione trial. Gruppo italiano per io studio della sopravvivenza nell'infarto miocardico. *Lipids* 36:S119-S126.

Noone EJ, Roche HM, Nugent AP, Gibney MJ (2002) The effect of dietary supplementation using isomeric blends of conjugated linoleic acid on lipid metabolism in healthy human subjects. *Br J Nutr* 88(3):243-251.

Ostrowska E, Muralitharan M, Cross RF, Bauman DE, Dunshea FR (1999) Dietary conjugated linoleic acids increase lean tissue and decrease fat deposition in growing pigs . *J Nutr* 129:2037-2042.

Pariza MW, Ha YL, Benjamin H, Sword JT, Gruter A, Chin SF, Storkson J, Faith N, Albright K. Formation and action of anticarcinogenic fatty acids. Adv Exp Med Biol 1991, 289:269-272

Roche HM, Noone E, Sewter C, Mc Bennett S, Savage D, Gibney MJ, O'Rahilly S, Vidal-Puig AJ. Isomer-dependent metabolic effects of conjugated linoleic acid: insights from molecular markers sterol regulatory element-binding protein-1c and LXRalpha. *Diabetes* 51(7):2037-44.

Ryder JW, Portocarrero CP, Song XM, Cui L, Yu M, Combatsiaris T, Galuska D, Bauman DE, Barbano DM, Charron MJ, Zierath JR, Houseknecht KL (2001) Isomer-specific antidiabetic properties of conjugated linoleic acid. Improved glucose tolerance, skeletal muscle insulin action, and UCP-2 gene expression. *Diabetes* 50(5):1149-1157.

Skerrett PJ, Hennekens CH (2003) Consumption of fish oils and decreased risk of stroke. *Prev Cardiol* Winter 6(1)38-41.

Thom E, Wadstein J, Gudmundsen O (2001) Conjugated linoleic acid reduces body fat in healthy exercising humans *J Int Med Res* 29(5):392-396.

West DB, Delany JP, Camet PM, Blohm F, Truett AA, Scimeca J (1998) Effects of conjugated linoleic acid on body fat and energy metabolism in the mouse. *Am J Physiol* 275:R667-R672.

Fisher, P. B. (1984) in Tumor Promotion and Cocarcinogenesis in Vitro: Mechanisms of Tumor Promotion, ed. Slaga, T. J., (CRC, Boca Raton, Fla.), vol. 3, pp. 57-123.

Bishop, J. M. (1991). Cell 64, 235-248.

Knudson, A. G. (1991). Proc. Natl. Acad. Sci. USA 90, 10914-10921.

MacLachlan, T. K., Sang, N. & Giordano, A. (1995). Crit. Rev. Eukaryotic Gene Express. 5, 127-156.

Sang, N., Baldi, A. & Giordano, A. (1995). Mol. Cell. Differ. 3, 1-29.

Y. L Ha, N K. Grimm and M. W. Pariza, in Carcinogenesis, Vol. 8, No. 12, pp. 1881-1887 (1987).

Y. L. Ha, N. K. Grimm and M. W. Pariza, in J. Agric. Food Chem., Vol. 37, No. 1, pp. 75-81 (1987).

Claims

Conjugated Linolenic Acid (CLnA™) compositions: synthesis, purification and uses

FIELD OF THE INVENTION

The invention relates to the field of human and animal nutrition, and more particular to novel compositions of conjugated linolenic acids compounds (CLnA™: a 1:1 mixture of C18:3 9*cis*,11*trans*,15*cis*-octadecatrienoic acid and C18:3 9*cis*,13*trans*,15*cis*-octadecatrienoic acid) and their derivatives (salts, amides, esters, adducts and complexes). More precisely, the invention relates to the process for their production and its use for prevent and/or treat different deceases like cancer, obesity, diabetes, atherosclerosis, cerebral vascular disease (CVD) and all related coronary artery deceases. These compositions are prepared from materials rich in alpha or gamma linolenic acids like linseed oil or evening primrose oil and more particularly from a new natural source *Plukenetia volubilis* (Sacha Inchi or Inca Peanut), a native plant of the high altitude rain forests of the Andean region of South America. According to this process, only water is used as a solvent for isomerisation with a metal alkali (i.e NaOH, KOH, $Ca(OH)_2$) as catalyst.

When linseed oil is used as starting material for execution of the present invention, the reaction produces approximately 30% of a 1:1 mixture of C18:3 isomers: 9*cis*,11*trans*,15*cis*-octadecatrienoic acid and 9*cis*,13*trans*,15*cis*-octadecadienoic; 9.5% of saturated fatty acids (5.4% palmitic and 4.3% stearic). The isomerised oil also contains 20% of unreacted oleic acid, 13% of unreacted linoleic acid (C18:2 9*cis*, 12*cis*); 4 % of CLA wherein 1.6% accounts for C18:2 9*cis*, 11*trans* and 2.3 % for C18:2 10*trans*, 12*cis*. The isomerised oil also contains 9% of unreacted linolenic acid (C18:3 9*cis*, 12*cis*, 15*cis*). All other full conjugated C18:3 compounds accounts for 9% and the cyclic compound C18:3 11,13 ciclohexadiene accounts for 6.7 %.

When *Plukenetia volubilis* (Sacha inchi) oil is used as starting material for execution of the present invention, the reaction also produces approximately 30% of a 1:1 mixture of C18:3 isomers: 9*cis*,11*trans*,15*cis*-octadecatrienoic acid and 9*cis*,13*trans*,15*cis*-octadecadienoic. Oleic

We claim:

Description

Field of the invention

This application relates to CLnA™ compounds, compositions and therapeutic methods for the treatment of cancers by administering CLnA™ compounds.

The invention relates to the discovery that CLnA™ compounds induce cytotoxicity by apoptosis of human cancer cells and therefore may be used for the treatment or prevention of cancer.

Background of the invention

Cancer is a complex multifactor and multistep process involving the coordinated expression and suppression of genes functioning as positive and negative regulators of oncogenesis (Fisher, 1984; Bishop, 1991; Knudson *et al.*, 1991; MacLachlan *et al.*, 1995; Sang *et al.*, 1995). Solid tumors are the leading cause of death attributable to cancers worldwide. Conventional methods of treating cancer include surgical treatments and the administration of chemotherapeutic agents. However, to date, such treatments have been of limited success. Chemotherapeutic treatments available today are also of limited usefulness because of their non-selective killing and / or toxicity to most cell types. Also, many tumor cells eventually become resistant against the chemotherapeutic agent, thus making treatment of solid tumors and other tumors non-feasible.

Cells can die either from apoptosis or necrosis. Unlike necrosis which is a pathological cell death, apoptosis is a death which is initially programmed in the gene of the cell itself. Thus, the gene which programs the apoptosis is activated by certain external or internal causes whereby programmed cell death gene protein is produced based upon said gene and then the cell itself is decomposed and dead by the resulting programmed death protein. Cells that undergo apoptotic cells death are characterized by a number of functional and morphologic changes: loss of membrane asymmetry, which results in the exposure of phosphatidylserine (PS) on the outer surface of cell membrane; loss of the inner mitochondrial membrane potential; activation of cytoplasmic serine proteases (caspases); rapid formation of extrusions of the cell membrane, which results in the formation of small extracellular membrane-coated particles (bleds); shrinkage of the total cell volume; condensation of the nuclear chromatin, which leads to the shrinkage of the nucleus, and fragmentation of the nucleus and the remaining cytoplasm into apoptotic bodies (Cohen, 1993).

Pharmaceuticals which have been used in clinical therapy include many agents such as anticancer agents, antibiotic substances, immunopotentiators, immunomodulators, etc. (such as alkylating agents antimetabolites and plant alkaloids) but it can be hardly said that such a drug therapy has been completely established already.

An object of the present invention is to develop a substance having a physiological function such as apoptosis-inducing action.

Conjugated linoleic acid (CLA) is a general term used to name positional and geometric isomers of linoleic acid C18:2(9 *cis*,12 *cis*). It usually denotes a mixture of mainly two isomers: C18:2(9*cis*, 11*trans*) and C18:2(10*trans*,12*cis*). They are usually present in a 1:1 ratio and the sum of these two isomers can vary between 30% and 90%. The majority of CLA in nutraceutical market do not mention the accurate composition for the content of each isomer, but generally the product is around 60% for both isomers. The most important isomer in term of activity is the C18:2(9*cis*, 11*trans*) (Seidel *et al.*, 2001, Patent. 6,319,950, Liu *et al.*, 2002, Roche *et al.*, 2002, Pariza *et al.*, 1991).

Conjugated linolenic acid is a general designation for fatty acids with 18 carbons and three doubles bonds (e.g. a triene). The invention relates to the discovery of a particular mixture of isomers of conjugated linolenic acid: CLnA™ C18:3(9*cis*,11*trans*,15*cis*) and C18:3(9*cis*,13*trans*,15*cis*). They are present in a 1:1 ratio and the sum of these two isomers may vary between 35% and 90% depending of the degree of purification.

The resemblance between the most important isomer of CLA [C18:2(9*cis*, 11*trans*)] and one of the isomers of CLnA™ [C18:3(9*cis*,11*trans*,15*cis*)] in term of their structure is the 9*cis*,11*trans* insaturation. We can say that this isomer has a "CLA characteristic". The major difference between both isomers is the third insaturation: 15*cis*. This insaturation confers a "omega-3 fatty acid characteristic". This should increase the bioavaibility of the product and therefore increase the activity of CLnA™. The aims of the current studies are intended to demonstrate the additive effects of these two characteristics (CLA and omega-3 fatty acid in the same molecule).

The free, naturally occurring CLA have been previously isolated from fried meats by Ha *et al.*, (1987). Since then, they have been found in some processed cheese products (Ha *et al.*, 1987). CLnA™ is naturally present as a minor component of cheese from cow milk (Winkler *et al.*, 2001) and bovine milk fat (Destaillats *et al.*, 2003).

CLA have been suggested as useful as anti-cancer agents for treatment of cancer. The latest research reveals the most dramatic impact may be on the reduced risk and incidence of mammalian cancer (breast and colon cancer). It has been shown that CLA down-regulated mammary growth, decrease the population and proliferation activity of the cancer cells, and therefore reduces mammary cancer risk and metastasis in mice and rats (Ha *et al.*, 1987, Ip *et al.*, 1999). The growth inhibitory effect of CLA was also demonstrated on human breast cancer cells (Durgam *et al.*, 1997).

Recent studies have shown that conjugated trienoic fatty acids reduce body fat, modulate triacylglycerols metabolism and serum and liver lipid levels (free fatty acids, cholesterol and lipoproteins) in rats (Koba *et al.*, 2002). Thus, CLnA™ could have beneficial effects in atherosclerosis and other cardiovascular diseases. The adipose tissue weight-reducing effect of conjugated trienoic fatty acids may be related to a decrease

either in triacylgycerol deposition from the circulation and / or in lipolysis in adipose tissues (Koba *et al.*, 2002).

Conjugated trienoic fatty acids have been suggested as useful compounds in the treatment of cell growth. Cytotoxic and anticarcinogenic effects of conjugated trienoic fatty acids have been shown on rat mammary carcinogenesis model (Futakuchi *et al.*, 2002, Tomoyuki *et al.*, in Patent No. JP2000336029). Same effects were observed on some lines of human tumor cells, possibly due to the induction of apoptosis of the cells (Igarashi *et al.*, 2000a,b). In all of these studies, the authors demonstrated some properties of conjugated trienoic fatty acids, but the structure, the geometrical and positional isomers of conjugated trienoic fatty acids responsible for these effects remain to be elucidated. CLnA™ may provide potent new therapeutic molecules for the treatment of disorders such as cancers.

Tomoyuki *et al.*, in Patent No. JP2000336029 relates to a new inhibiting agent useful in food and medicinal fields by incorporating a conjugated linolenic acid. This breast cancer-inhibiting agent contains a conjugated linolenic acid (e.g. 9,11,13-octadecatrienic acid, 10,12,14 octadecatrienic acid, their mixtures.). The breast cancer-inhibiting agent can be used not only as a medicine but also as a breast cancer-inhibiting or preventing food (e.g. a conjugated linolenic acid-containing oil and fat product), and in both cases of usage, the conjugated linolenic acid to be ingested is generally 0.01-3%, preferably 0.05-1% of the food weight.

Horrobin *et al.*, in US Patent No. 6,245,811 disclosed a method for treating a disorder like complications of cancer; with compounds of structure containing group like CLA, as fatty esters as bioactive compounds

Seidel *et al.*, in US Patent No. 6,319,950 disclosed a method for the treatment of carcinoma in a human, including administering to a human a therapeutically effective amount of C18 (9-*cis*, 11-*trans*). This patent includes administering to a human a purified conjugated linoleic acid (CLA) produced by a novel synthesis process for producing C18 (9-*cis*, 11-*trans*).

Das *et al.*, in US Patent No. 6,426,367 disclose methods of selectively reducing the blood supply to a neoplastic region, such as a tumor region, thereby selectively causing necrosis of the neoplastic tissue without substantial necrosis of adjoining tissues. The methods described in this patent employ intra-arterial injection of polyunsaturated fatty acids, such as CLA, preferably in the form of salts, preferably with a lymphographic agent, and optionally with an anti-cancer drug, and/or a cytokine.

Das *et al.*, in US Patent No. US2002077317 disclosed a method of stabilizing and potentiating the actions of 2-methoxyoestradiol, statins, H2 blockers, and C-peptide of proinsulin which have modified influence on angiogenesis and inhibiting the growth of tumor cells, as applicable by using in coupling conjugation certain polyunsaturated fatty acids (PUFAs) chosen from linoleic acid, gamma-linolenic acid, dihomo-gamma-linolenic acid, arachidonic acid, alpha-linolenic acid, eicosapentaenoic acid,

docosahexaenoic acid, *cis*-parinaric acid or conjugated linoleic acid in predetermined quantities.

Bin *et al.* in Patent No. CN1371985 disclosed a health-care wine containing conjugated linoleic acid or conjugated linoleic acid derivative. Said wine not only has the features of general drinking wine, but also possesses the health-care functions of resisting cancer, resisting atherosclerosis, regulating and controlling metabolism, raising immunity, regulating blood sugar and promoting growth development.

Bin *et al.*, in Patent No. CN1356386 disclosed a process for preparing conjugated linoleic acid from dewatered castor oil includes physicochemically induced isomerizing, hydrolysis and multi-step separation. The resultant product contains conjugated linoleic acid (higher than 80%), linoleic acid (higher than 15%) and their isomers. It features its functions of preventing and treating cancer, diabetes and atherosclerosis, improving immunity, reducing blood sugar and fat.

Focant *et al.*, in Patent No. WO02051255 relates to methods for altering the fatty acid composition in milk or tissue fat directly derived from a milk producing ruminant. In this patent methods are disclosed to obtain said desirable fatty acid profile, thereby improving the nutritional benefits to human health associated with CLA. Dietary intakes of CLA [C18:2 *cis*-9, trans-11] and C18:1 *trans*-11 fatty acids in milk or meat, or products thereof, produced in accordance with this invention in ruminant animals, can be effective in preventing cancer in different sites, reduce risk of coronary heart disease and to enhance immune function.

Summary of the invention

CLnA™ compounds were synthesized and analyzed for therapeutic activities, including anti-cancer activities, against human breast cancer cells lines. CLnA™ compounds of the invention were found to possess potent inhibitory activities affecting cancer cell proliferation and survival. CLnA™ compounds of the invention are demonstrated as useful for the treatment of human breast tumors, inducing cytotoxicity of human cancer cells by apoptosis.

Description of the figures

Figure 5: Cytotoxicity of CLA (100 μM) on MDA-MB-231 cells

Figure 6: Cytotoxicity of CLnA™ (100 μM) on MDA-MB-231 cells

Figure 7: Cytotoxicity dose-dependant of CLA on MDA-MB-231 cells

Figure 8: Cytotoxicity dose-dependant of CLnA™ on MDA-MB-231 cells

Figure 9: Apoptosis induced by CLA (100 μM) on MDA-MB-231 cells

Figure 10: Apoptosis induced by CLnA™ (100 μM) on MDA-MB-231 cells

Figure 11: Fluorescence microscopy of apoptosis induced by CLnA™ (100 μM) on MDA-MB-231 cells

Description of the invention

Toward that end, the inventors, have discovered that CLnA™ induce cytotoxicity of human cancer cells by apoptosis. The method of the present invention provides for the treatment of cancer in a human, including the treatment of mammary cancer. The method of the present invention provides cytotoxicity of cancer cells using CLnA™. CLnA™ has a significant potency relative to other fatty acids in respect to an ability to modulate tumorigenesis.

Methods of treatment

The compounds of the invention are useful for the treatment of human cancer cells. In particular, the compounds of the invention have been found to be potent inhibitors of tumor cell proliferation and survival, and effective to induce apoptosis of malignant human cells. Compounds of the invention have been found to be effective for inducing cytotoxicity and / or apoptosis of human breast cancer cells.

Examples

The invention may be further clarified by reference to the following Examples, which serve to exemplify some of the preferred embodiments, and not to limit the invention in any way.

Example 1

Synthesis of CLnA™

Example 2

Cytotocixity of CLA and CLnA™ compounds

The cytotoxicity of the CLA and CLnA™ compounds against two human tumor cells lines was evaluated. The CLA and CLnA™ compounds, prepared as described above, were tested, along with 1 % (v/v) of ethanol in culture medium as a control.

Cell Culture

Human cell lines were obtained from American Type Culture Collection (ATCC,

Rockville, MD). Cells used in this study include estrogen receptor negative human breast cancer cells MDA-MB-231 and estrogen receptor positive MCF-7. They are cultured in a humidified 5 % CO_2 atmosphere, at 37 degree C. Cells were maintained as a continuous cell line in Modified Eagles' medium supplemented with 10 % fetal bovine serum, and antibiotics.

MTT proliferation assay

The cytotoxicity of various compounds against human tumor cell lines was performed using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay (Sigma Chemical Co., St. Louis, MO). Briefly, exponentially growing tumor cells were seeded into a 96-well plate at a density of 1500 cells / well and incubated for 4 hours at 37 °C prior to drug exposure. For the treatment, culture medium was carefully aspirated from the wells and replaced with fresh medium containing the vehicle (ethanol 1 %, (volume in culture medium), CLA or CLnA™ compounds at concentrations ranging from 10 to 100 µM. Fatty acids were complexed to bovine serum albumin (BSA) 1 h at 37 °C with agitation, prior to be added to the cells. Triplicate wells were used for each treatment. The cells were incubated with the various compounds for 24-96 hours at 37 °C in a humidified 5% CO_2 atmosphere.

After incubation, cell survival was determined using a tetrazolium (MTT)-based colorimetric assay (Mosmann, *et al.*, 1983). Briefly, MTT assay mesure the cell proliferation related to the mitochondrial activity. In a viable cells, there are active mitochondrias that reduce the yellow compound MTT in a blue compound. To each well, 100 µL of MTT (0.5 mg/ml final concentration in phosphate buffered saline) was added and the plates were incubated at 37 °C for 4 hours in a humidified 5% CO_2 atmosphere to allow MTT to form formazan crystals by reacting with metabolically active cells. The formazan crystals were solubilized in a solution containing 10 % SDS in 0.01 M HCL, for 3 h at 37 °C in a humidified 5% CO_2 atmosphere. The optical absorbance of each well was measured in a microplate reader spectrophotometer (Synergy HT, Biotek) at 570 nm and a reference wavelength of 630 nm. The percent cytotocixity was calculated using the formula: 1-(x570/xctrl) X100. Each experiments was done in triplicate and repeated 3 times.

Detection of apoptosis and necrosis by fluorescence-based microplate

Exponentially growing cells were seeded in 96-well tissue culture plates at a density of 1500 cells / well and cultured for 36 hours at 37 °C in a humidified 5% CO_2 atmosphere. The supernatant culture medium was carefully aspirated and replaced with fresh medium containing the vehicle (ethanol 1 % v/v), CLA or CLnA™ compounds at concentrations ranging from 10 to 100 µM. After incubation, apoptosis and necrosis was determined by adding fluorescence markers of cell death: 50 µL of staining solution (YO-PRO-1 5 µg/mL and PI 20 µg/mL, Molecular Probes) is added to each well. YO-PRO-1 is a specific dye for apoptotic cells while propidium iodide (PI) is a specific dye for necrotic cells. YO-PRO-1 dye is permeant to apoptotic cells, providing a convenient indicator of apoptosis. There is selective uptake of YO-PRO-1 by apoptotic cells. YO-PRO-1 nucleic acid stain selectively passes through the plasma membranes of apoptotic cells and

labels them with green fluorescence. Necrotic cells are stained with the red-fluorescent PI, a DNA-selective dye that is membrane impermeant but that easily passes through the compromised plasma membranes of necrotic cells. Live cells are not stained by either YO-PRO-1 or PI. Plates were then incubated in dark for 30 min on ice. Fluorescence was measured with a microplate spectrophotometer (Synergy HT, Biotek). Each experiments was done in triplicate and repeated 3 times.

Detection of apoptosis and necrosis by Fluorescence Microscopy

In brief, 10X 5 cells / ml were grown for 48 h on glass coverslips placed in 6-well plates with media containing 100 µM CLnA™ or ethanol 1 % as control. Cells were washed twice with binding buffer (10 mM HEPES, 140 mM $NaCl_2$, 2.5 mM $CaCl_2$, pH 7.4). Cells were then incubated in the dark with annexin V conjugated to fluorescein isothiocyanate (FITC, Molecular Probes) and 0.20 µg/ml PI for 20 min at room temperature. After washing twice the cells with buffer, the coverslips were mounted onto slides with Vectashield (Vector Labs, Burlingame, CA) and viewed with a fluorescence microscope. Cells were visualized and photographed at a primary magnification of 40 times. Each experiments was done in triplicate and repeated 3 times.

A characteristic of apoptotic cells is the translocation of PS residues, that are normally confined to the inner leaflet of the plasma membrane, to the outer leaflet (Martin *et al.*, 1995). This plasma membrane change can be efficiently detected by the use of FITC-conjugated annexin V, a protein with extremely high affinity for binding to PS, and observation of cells by fluorescence microscopy. FITC-labeled annexin V was used to bind exposed PS on cells undergoing the early stages of apoptosis. Annexin V will selectively bind these exposed PS. PI is membrane impermeant and bind to DNA by intercalating between bases. PI also binds to RNA. Once the dye is bound to nucleic acids, its fluorescence is enhanced. PI is excluded from viable cells and fluoresces red in the presence of DNA. In the color photographs, red fluorescence represents nuclei stained with PI. Green or yellow (e.g. superimposed red plus green) represents the apoptotic cells. Non-apoptotic cells do not incorporate significant amounts of PI, and consequently have much less fluorescence than apoptotic cells. Using a combination of these fluochromes it was possible to distinguishes between viable cells (do not incorporate neither annexin V nor PI), early apoptotic (green fluorescence), late apoptotic (green fluorescence with red fluorescence) and necrotic cells (red fluorescence).

Results

Effect of CLA or CLnA™ *on Proliferation of human breast cancer cell lines*

Two human breast cancer cell lines, the MDA-MB-231 and MCF-7 were treated with CLA or CLnA™ at concentrations of 10 to 100 µM for 24 to 96 hours or with ethanol 1% (v/v) as a control. Our results demonstrated that when MDA-MB-231 cells were incubated with CLA 100 µM for different period of time, there is an increase in the cytotocixity of the cells (Fig. 5). After 96 h, about 70 % of cell death.

When the MDA-MB-231 cells were treated with CLnA™ 100 µM, there is also an increase in the cytotocixity of the cells. After 96 h, almost all the cancer cell are dead. (Fig.6). The same results were also observed on MCF-7 cells. From these results, we can conclude that CLnA™ is more cytotoxic on human cancer cell than CLA.

CLA and CLnA™ were shown to inhibit the proliferation of breast cancer cell lines in a dose-dependent manner. As can be seen from the results in Fig. 7 and 8, maximum inhibition of cell proliferation occurred at 100 µM CLA or CLnA™. The same results were also observed on MCF-7 cells. These results provide evidence that a compound according to the invention, CLnA™, effectively inhibits dose-dependently the proliferation of human breast cancer cells.

Apoptosis or necrosis

Cells can died either from apoptosis or necrosis. We determine which death mechanism is induced by CLA and CLnA™. For this purpose, we used fluorescence markers of cell death:YO-PRO1 is a specific dye for apoptotic cells while PI is a specific dye for necrotic cells.

When MDA-MB-231 cells were treated with CLA 100 µM for different period of time, there is a small increased in apoptosis (Fig. 9). When MDA-MB-231 cells are treated with CLnA™ 100 µM, there is a significant increase in the fluorescence of YO-PRO1 dye of apoptosis (Fig.10). No necrosis was induced by CLnA™. The same results were also obtain in MCF-7 cells.

Fluorescence microscopy

Apoptotic cells can be identified by PS exposure. Annexin V specifically bind to translocated PS. The hydrophilic dye PI has a high affinity for DNA but cannot pass the intact cell membrane (Nicolletti *et al.*, 1991). PS exposure in the absence of PI is generally held as a characteristic for early apoptotic cells when only minor morphologic changes are detectable. In contrast, cells stained with both annexin V and PI have lost their membrane integrity and are considered to be late apoptotic or necrotic cells.
Using annexin V as a FITC conjugate in combination with PI as an exclusion dye for cell viability, this assay can detect apoptotic cells and discriminate between apoptosis and necrosis (Vermes *et al.*, 1995). The annexin assay distinguished among early apoptosis, late apoptosis and apoptotic or necrotic phase in which the cells were labeled with both annexin V and PI. During early apoptosis, a loss of membrane asymmetry occurs when the PS is exposed on the outer leaflet of the plasma membrane. Annexin V will preferentially bind to PS and can therefore be used as an early indicator of apoptosis. In addition, PI can be used to assess plasma membrane integrity and cell viability. PI fluoresces red when bound to DNA or RNA, but is excluded from cells with intact plasma membranes.

In figure 11, the green fluorescence represented the externalization of PS residues and was indicative of apoptotic cultures. The results of annexin V-FITC binding studies further substantiated the fact that CLnA™ induced cell death in human breast cancer cells is a result of an apoptotic cell death mechanism rather than a necrotic pathway. As the

plasma membranes of cells become increasingly more permeable during the mid and late stage of apoptosis, PI becomes increasingly capable to penetrate the cells and staining nuclear DNA, producing a yellow red fluorescence signal.

In conclusion, CLnA™ is more cytotoxic than CLA on human breast cancer cell MDA-MB-231 and MCF-7. CLnA™ induce about 96 % of cytotoxicity while CLA induce about 70 % of cell death. CLnA™ is more apoptotic than CLA by at least 2 times. CLnA™ induced no necrosis. We also demonstrated that CLnA™ induced cell death by apoptosis with the use of annexin V and PI dyes. Both the MTT proliferation assay and the fluorescence assay showed that CLnA™ could inhibit cancer cells proliferation. The induction of apoptosis in human breast cancer cells suggest that CLnA™ could be used as a potential source of anti-cancer agents. Based on the foregoing results, it can be seen that CLnA™ has significant therapeutic application in the treatment or prevention of human cancers such as breast cancer, especially based on its inhibition of cancer cell proliferation and the induction of cancer cell apoptosis.

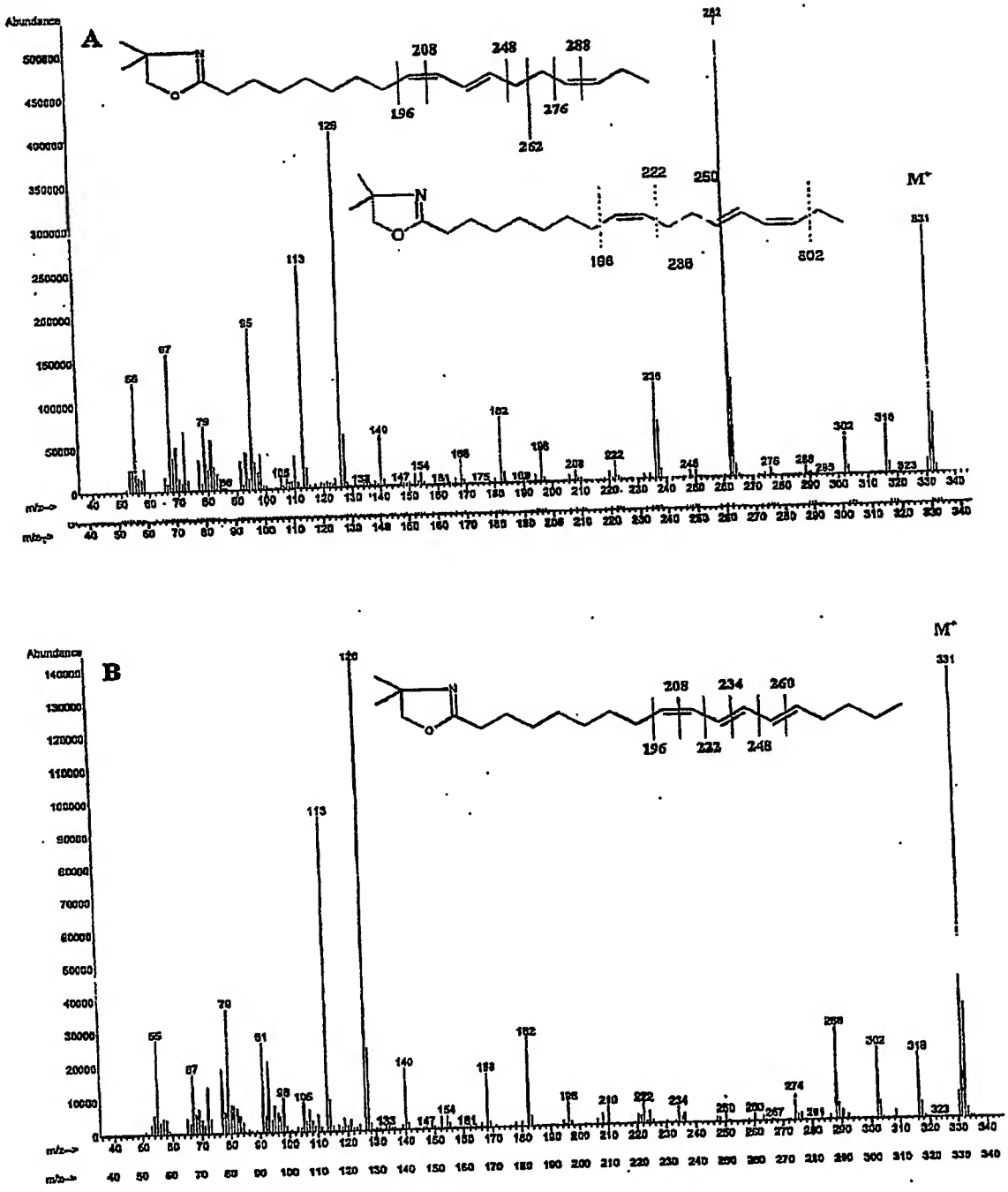


FIG. 1

Fig. 1: presents mass spectra of produtcs resulting of the isomerization process of alpha-linolenic acid (9Z,12Z,15Z-C18:3 acid), as 4,4-dimethyloxazoline derivatives: A, 9Z,11E,15Z and 9Z,13E,15Z -C18:3; B, 9,11,13-C18:3, C, 10E,12Z,14E-C18:3 and D, 11,13-CCLA (9-(6-propyl-cyclohexa-2,4-dienyl)-nonanoic acid).

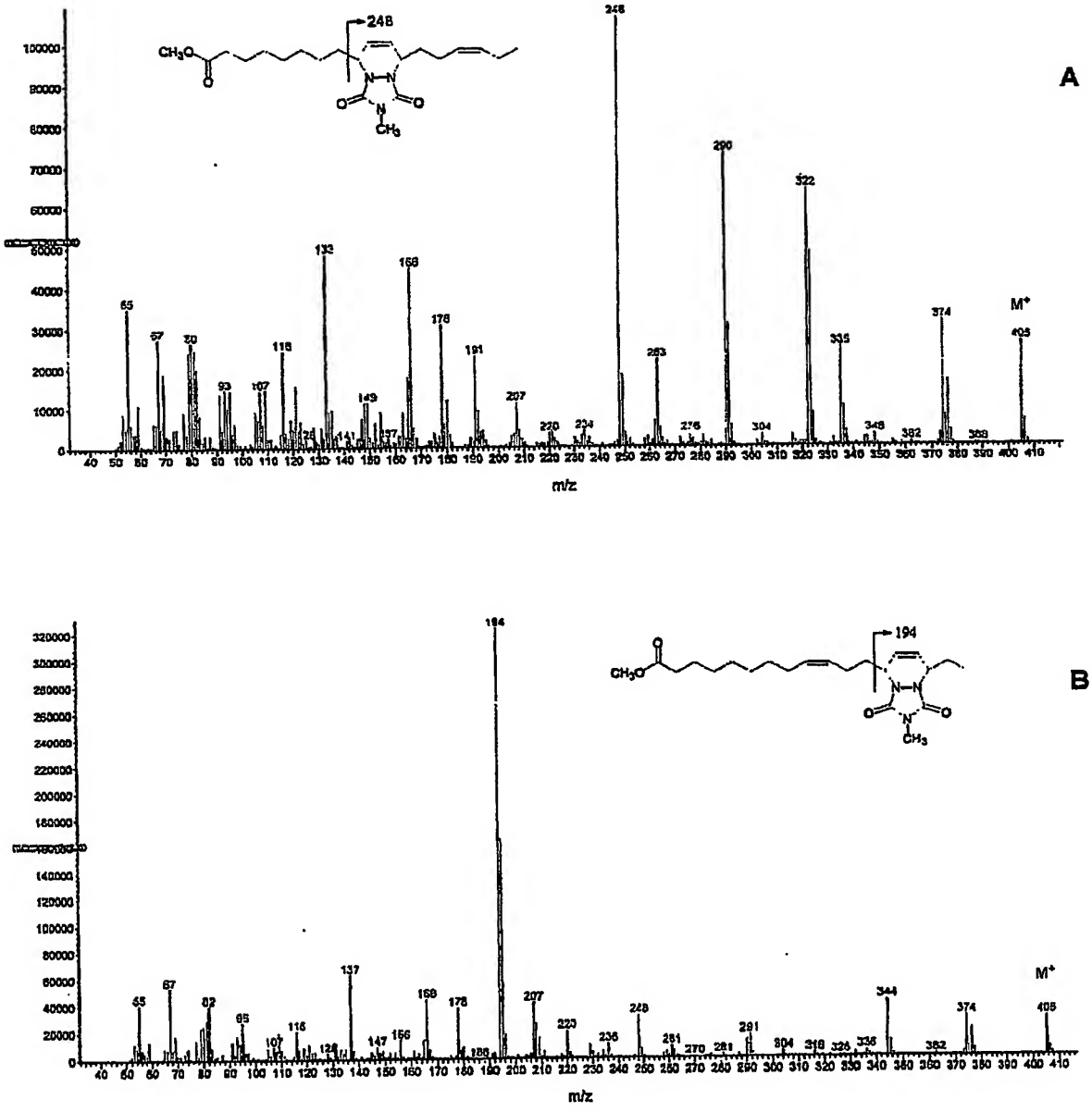
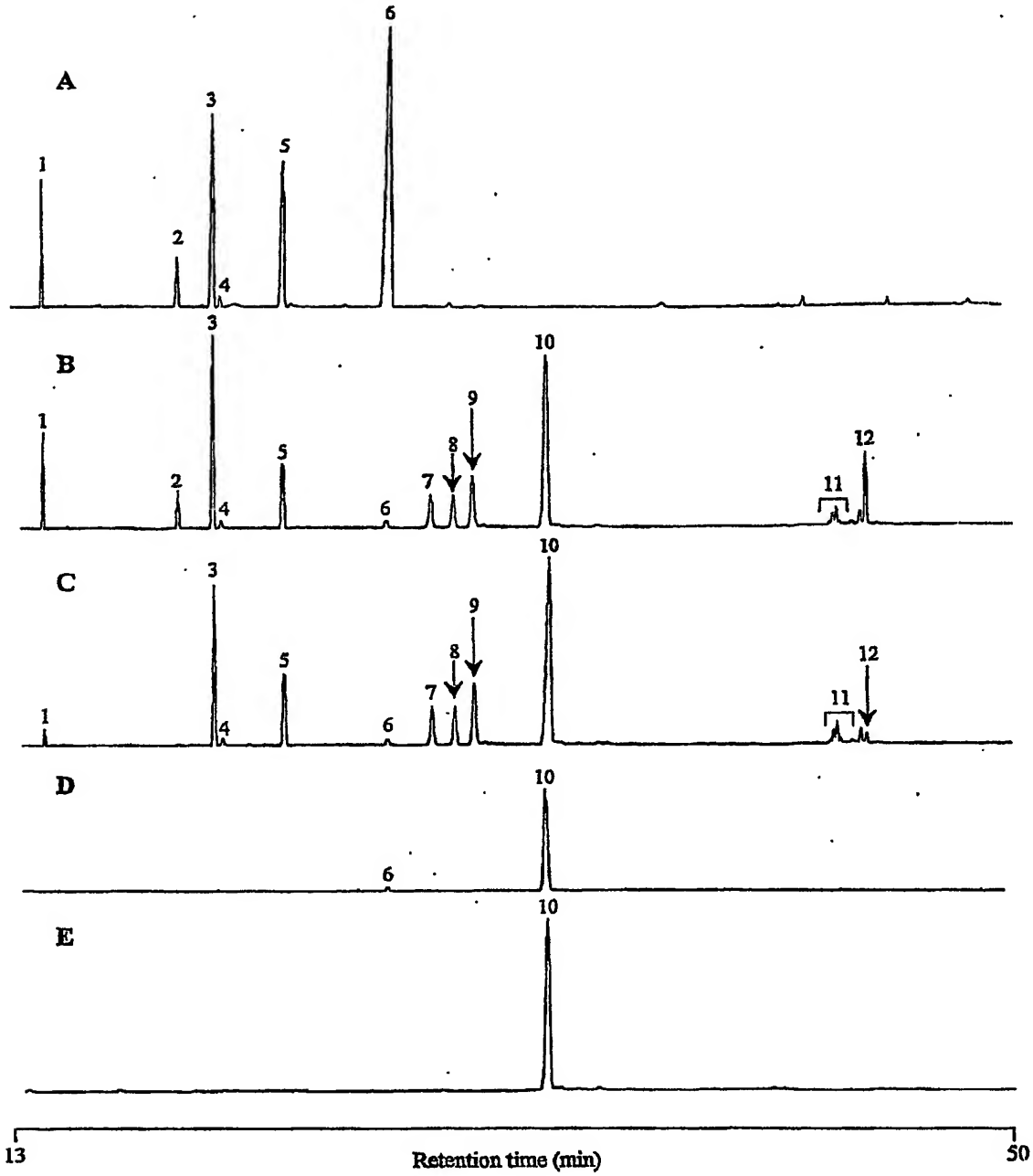


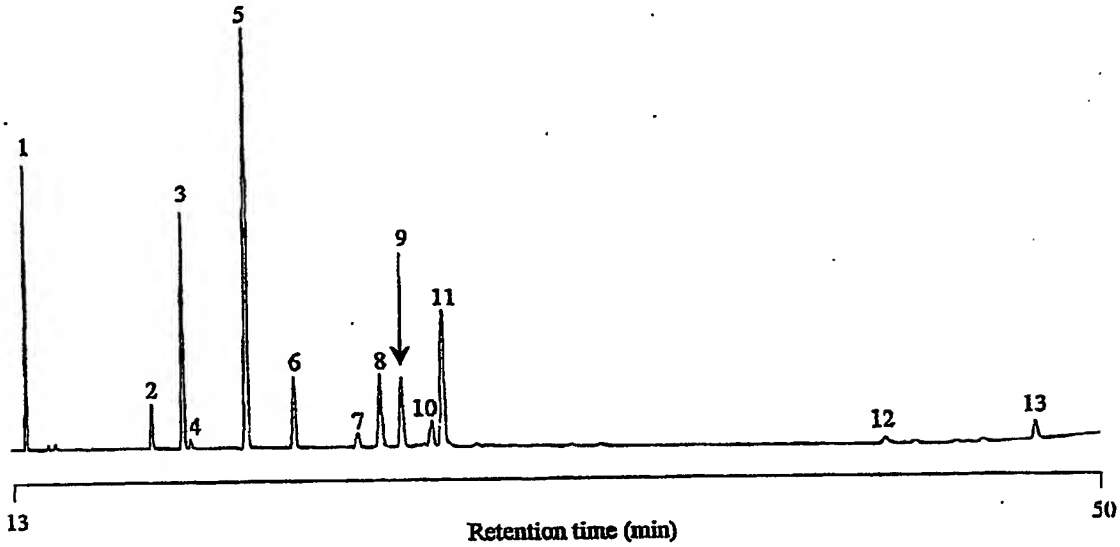
Fig 2: Mass spectra of the MTAD adducts of *cis*-9,*trans*-11,*cis*-15 18:3 (A) and *cis*-9, *trans*-13,*cis*-15 18:3 (B) acid methyl esters.

Fig. 3: present the thermal mechanism leading to the formation of 11,13-CCLA [9-(6-propyl-cyclohexa-2,4-dienyl)-nonanoic acid (Fig 1-D)] from 10E,12Z,14E-C18:3 acid.



1. Palmitic Acid
2. Stearic Acid
3. Oleic Acid
4. 11Z-C18:1 Acid
5. Linoleic Acid
6. Linolenic Acid
7. 9Z,11E-C18:2 Acid
8. 10E,12Z-C18:2 Acid
9. 9-(6-propyl-cyclohexa-2,4-dienyl)-Nonanoic Acid
10. 9Z,11E,15Z and 9Z,13E,15Z -C18:3 Acid
11. 9,11,13-C18:3 Acid
12. 10E,12Z,14E-C18:3 Acid

Fig. 4: present gas liquid chromatograms of fatty acid methyl esters obtained after methylation of linseed oil (A), conjugated linseed oil (B) liquid phase from urea crystallization (C), reversed-phase liquid chromatography fraction containing about 97 % of 9Z,11E,15Z-C18:3 acid (D), argentation liquid chromatography fraction containing about 99+ % of 9Z,11E,15Z and 9Z,13E,15Z -C18:3 acid (E)



1. Palmitic Acid
2. Stearic Acid
3. Oleic Acid
4. 11Z-C18:1 Acid
5. Linoleic Acid
6. Linolenic Acid
7. 6-(6-hexyl-cyclohexa-2,4-dienyl)-hexanoic Acid
8. 9Z,11E-C18:2 Acid
9. 10E,12Z-C18:2 Acid
10. 9Z-C20:1 Acid
11. 6Z,8E,12Z-C18:3 Acid
12. 9Z-C22:1 Acid
13. 7E,9Z,11E-C18:3 Acid

Fig 5: Gas liquid chromatogram of the fatty acid methyl esters obtained after methylation of partially conjugated evening primrose oil.

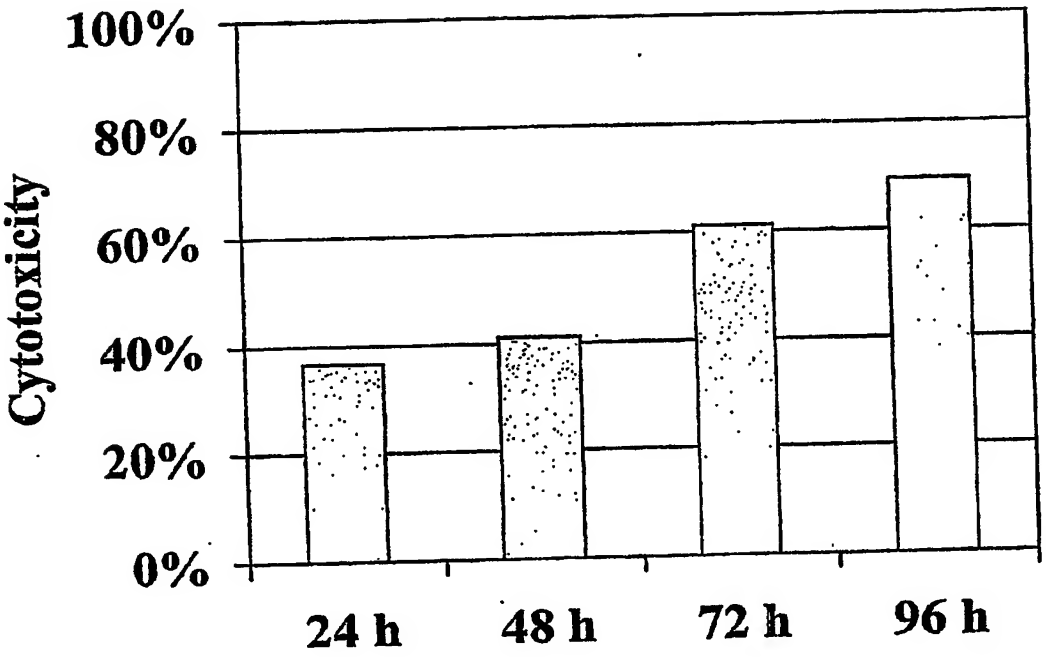


Figure 5: Cytotoxicity of CLA (100 μM) on MDA-MB-231 cells. Cells were exposed to 100 μM CLA for different period of time. Cytotoxicity was determined by colorimetric MTT cell proliferation assay as described in Methods. Results are shown as mean of triplicate experiments. This is one of the representative results of 3 independant experiments.

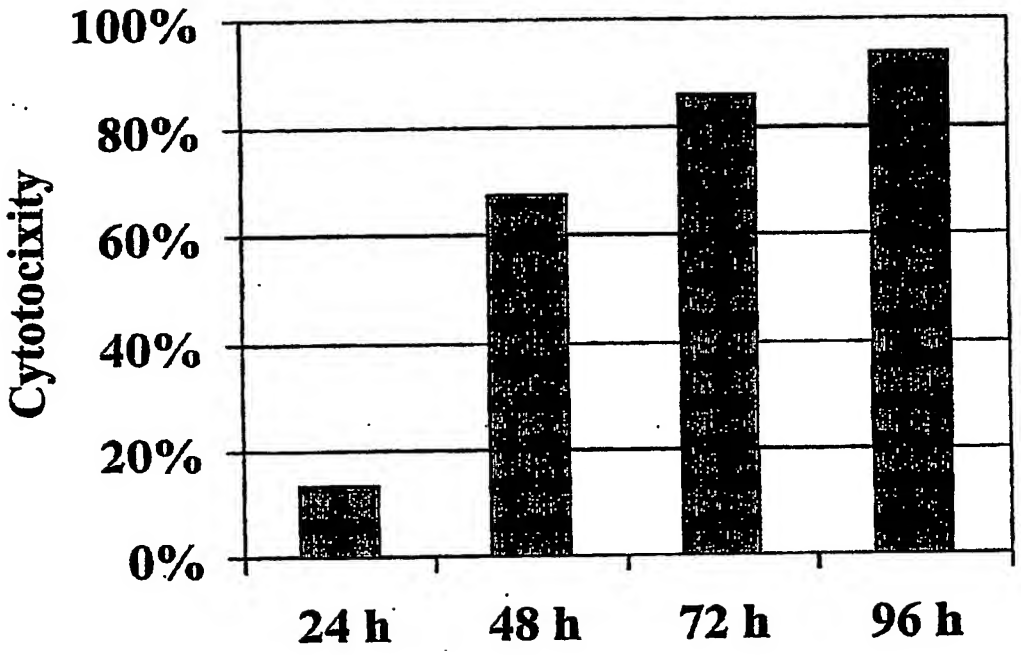


Figure 6: Cytotoxicity of CLnA™ (100 μM) on MDA-MB-231 cells. Cells were exposed to 100 μM CLnA™ for different period of time. Cytotoxicity was determined by colorimetric MTT cell proliferation assay as described in Methods. Results are shown as mean of triplicate experiments. This is one of the representative results of 3 independant experiments.

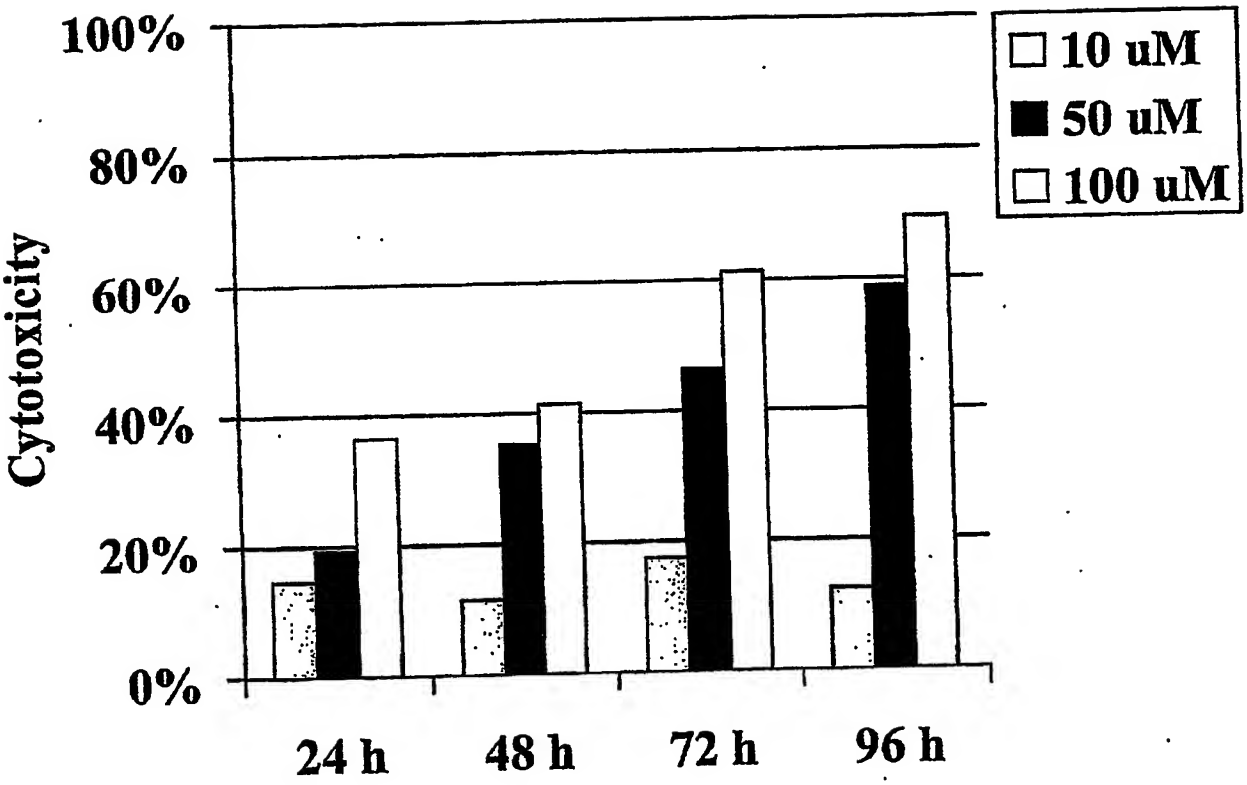


Figure 7: Cytotoxicity dose-dependant of CLA on MDA-MB-231 cells. Cells were exposed to different concentration of CLA for different period of time. Cytotoxicity was determined by colorimetric MTT cell proliferation assay as described in Methods. Results are shown as mean of triplicate experiments. This is one of the representative results of 3 independant experiments.

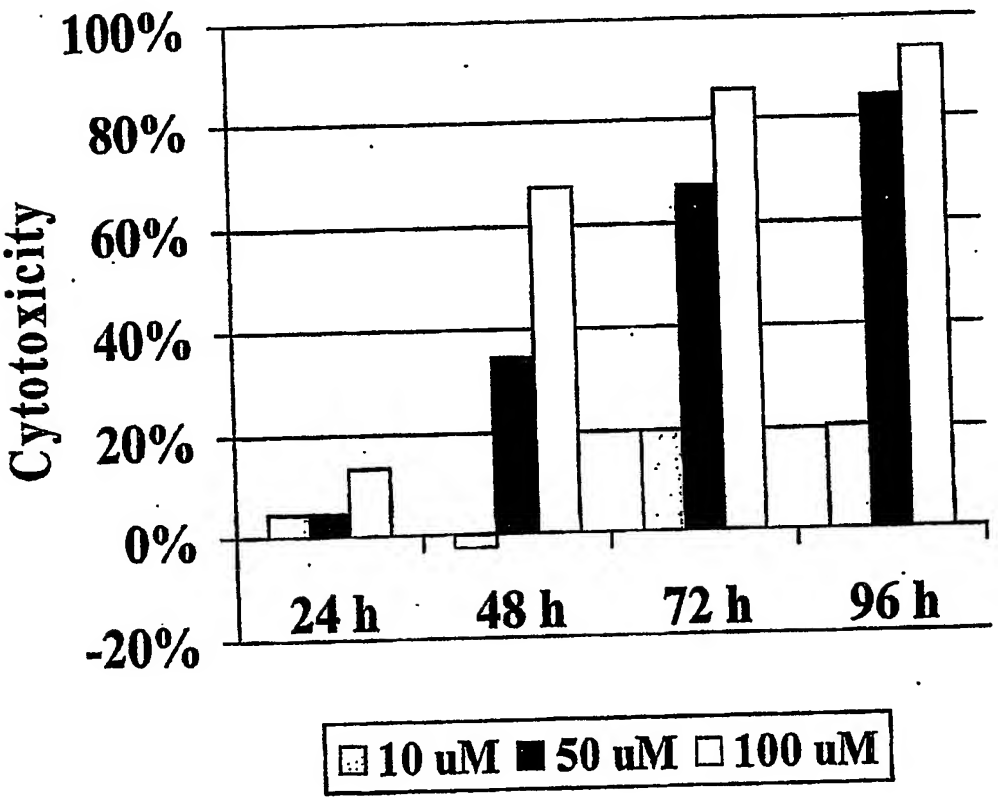


Figure 8: Cytotoxicity dose-dependant of CLnA™ on MDA-MB-231 cells. Cells were exposed to different concentrtion of CLnA™ for different period of time. Cytotoxicity was determined by colorimetric MTT cell proliferation assay as described in Methods. Results are shown as mean of triplicate experiments. This is one of the representative results of 3 independant experiments.

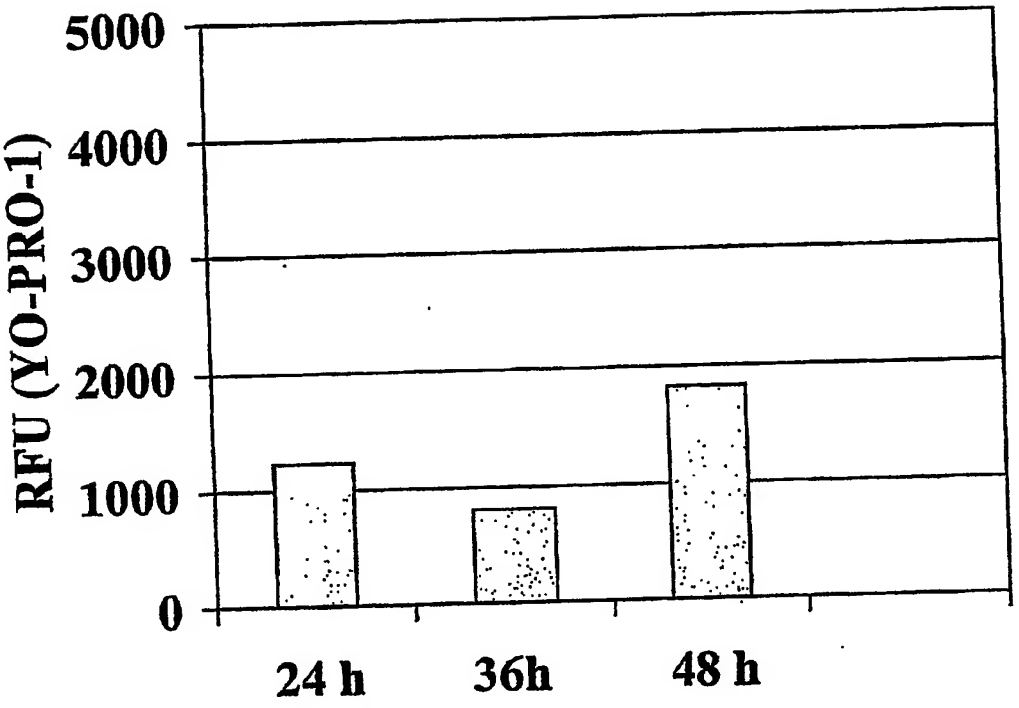


Figure 9: Apoptosis of CLA (100 μM) on MDA-MB-231 cells. Cells were exposed to 100 μM CLA for different period of time. Fluorescence of apoptotic cells was mesured by YO-PRO-1 dye as described in Methods. Results are shown as mean of triplicate experiments. This is one of the representative results of 3 independant experiments.

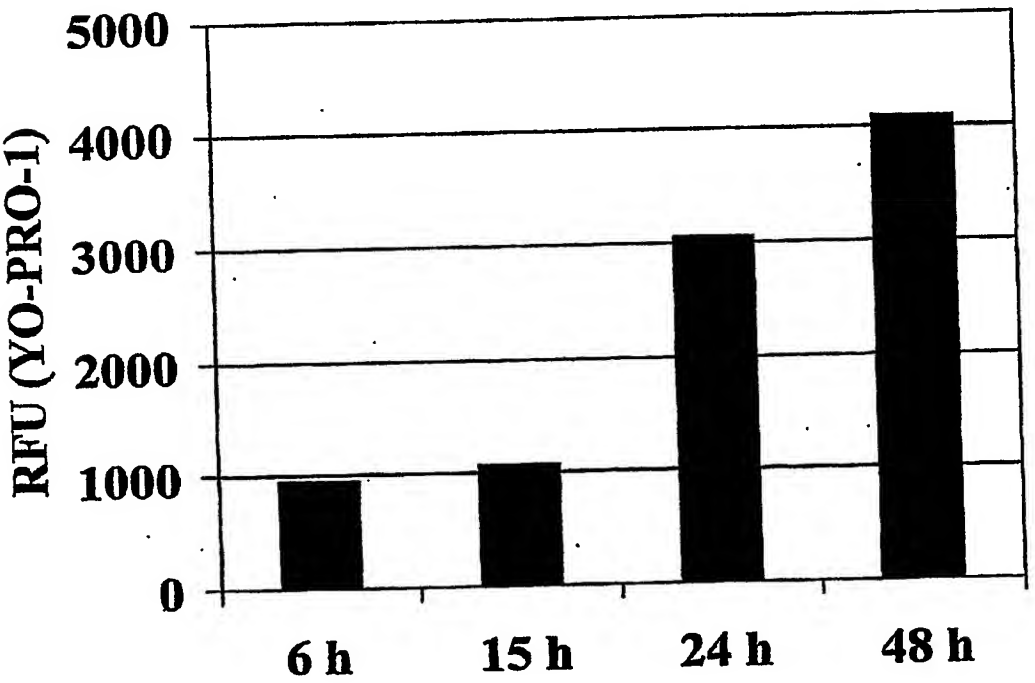


Figure 10: Apoptosis of CLNA™ (100 μM) on MDA-MB-231 cells. Cells were exposed to 100 μM CLnA™ for different period of time. Fluorescence of apoptotic cells was mesured by YO-PRO-1 dye as described in Methods. Results are shown as mean of triplicate experiments. This is one of the representative results of 3 independant experiments.

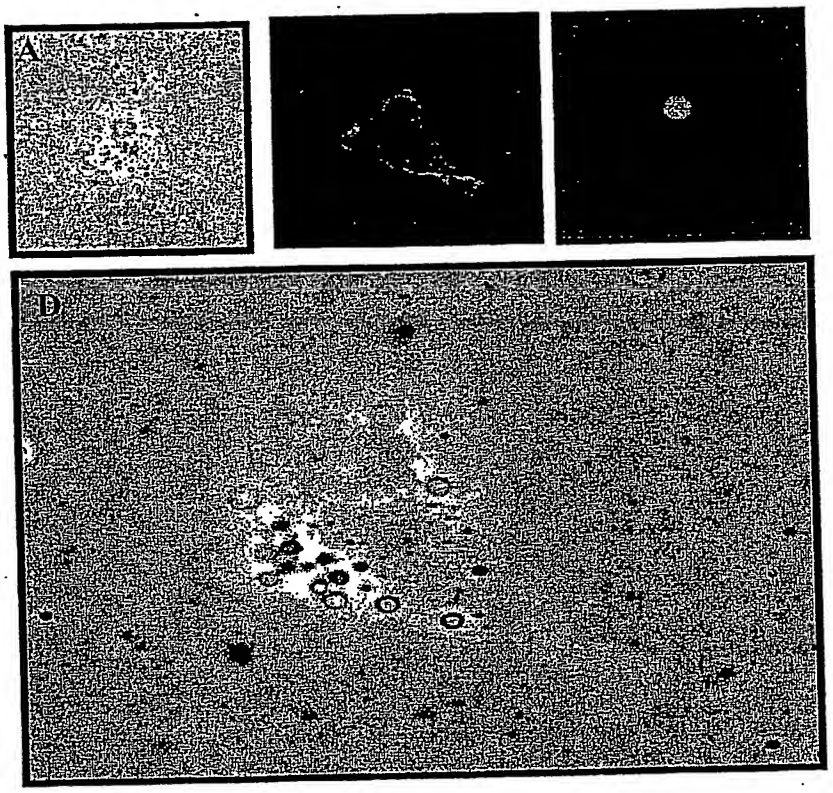


Figure 11: Apoptosis induced by CLnA™ (100 μM) on MDA-MB-231 cells. Cells were exposed to 100 μM CLnA™ for 48 h. Fluorescence was mesured using annexin V and PI dye as described in Methods. **A:** Photograph of MDA-MB-231 cells. Photographs were taken for annexin V (**B**) and PI (**C**). **D** is suremposition of the 3 photographs. Nuclei of apoptotic cell (green fluorescence) can be distinguished easily by PI red staining. This is one of the representative results of 3 independant experiments.